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**Development of fed-batch and perfusion strategies for intensified influenza
virus production in cell culture processes**

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Development of fed-batch and perfusion strategies for intensified influenza virus production in cell culture processes

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DEDICATION

To the Cristina of the past, for making the choices that led to this moment;

To the Cristina of the present, for her dedication and lightheartedness;

To the Cristina of the future, for trusting the other two.

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RÉSUMÉ

Les procédés de culture cellulaire sont de plus en plus considérés comme une alternative plus flexible, plus évolutive et plus réactive que les systèmes traditionnels à base d'œufs pour la production de vaccins contre le virus influenza. Pour devenir une plateforme rentable, les processus de production de virus à partir de cultures cellulaires doivent être intensifiés, ce qui, jusqu'à présent, a été réalisé presque exclusivement par le développement d'opérations en mode perfusion. Si les procédés en mode perfusion permettent généralement d'obtenir des densités cellulaires plus élevées, et donc une meilleure productivité, les volumes importants de milieu de culture consommés entraînent des défis au niveau de la préparation et de la gestion des courants d'entrée et de sortie, notamment dans les opérations à grande échelle. Dans ce contexte, l'objectif principal de cette thèse était de développer des stratégies d'alimentation efficaces pour intensifier la production de virus influenza dans des processus de culture cellulaire visant à minimiser la consommation de milieu de culture. Pour ce faire, des stratégies d'alimentation en modes fed-batch, perfusion et hybride ont été développées sur la base d'une approche simplifiée permettant d'évaluer le métabolisme général des cellules en fonction de la consommation des principaux nutriments.

Dans la première partie de ce travail, nous avons développé des procédés pour intensifier la production de virus influenza dans des cultures à haute densité de cellules HEK293. En utilisant des spin tubes comme modèle à petite échelle, nous avons conçu un mode d'opération hybride fed-batch/perfusion et un mode perfusion basée sur : i) une stratégie fed-batch dynamique, dans laquelle l'ajout de milieu de culture concentré a été effectué en fonction de la croissance cellulaire et du profil métabolique principal des cellules; ii) une stratégie de perfusion, dans laquelle le milieu de perfusion a été enrichi avec du milieu de culture concentré afin de réduire le taux de perfusion requis. Les processus établis ont ensuite été transposés à un bioréacteur couplé à un module de filtration en profondeur à flux tangentiel (*Tangential Flow Depth Filtration*, TFDF) comme dispositif de rétention des cellules. Ces stratégies ont permis de produire efficacement dans des cultures infectées entre $8-10 \times 10^6$ cellules/mL, résultant en une augmentation de plus de 4 fois de la production totale et de 3 fois du rendement espace-temps (space time yield, STY), par rapport à une culture référence opérée en mode batch et à faible densité cellulaire. Nous avons ainsi démontré que les stratégies d'alimentation qui minimisent la consommation de milieu de culture sont adaptées à la fabrication de virus influenza à grande échelle.

Dans la deuxième partie de cette thèse, nous intensifions la production de virus influenza dans des cultures de cellules MDCK en suspension, l'un des substrats cellulaires les plus utilisés pour la biofabrication de ce virus. L'objectif principal de cette section était d'évaluer si les stratégies établies pour les cellules HEK293 pouvaient être transposées à la production du même virus en utilisant une lignée cellulaire différente. La stratégie d'alimentation dynamique a été appliquée à des opérations hybrides fed-batch/perfusion et fed-batch conventionnelles pour la production de virus influenza dans des cultures de cellules MDCK à haute densité cellulaire. Alors que les opérations en mode fed-batch conventionnel ont conduit à une réduction de 20% du rendement spécifique en virus (*cell specific virus yield*, CSVY) pour les cultures infectées à 10×10^6 cellules/mL, l'opération en mode hybride a permis des productions de virus réussies dans les cultures infectées jusqu'à 15×10^6 cellules/mL. Lorsqu'appliqué à un bioréacteur couplé à un module de membrane TFDF pour la rétention des cellules, le procédé hybride a permis d'augmenter de plus de 8 fois la production totale et de plus de 7.4 fois le rendement espace-temps par rapport à une culture référence en mode batch.

Dans la dernière partie de cette recherche doctorale, nous avons évalué si les procédés développés pour la production de virus influenza pouvaient être appliqués pour intensifier la production d'autres virus. Au cours des dernières décennies, l'utilisation de procédés de culture cellulaire pour produire des vecteurs viraux à des fins de vaccination et de thérapie génique a suscité un intérêt croissant. Nous avons donc appliqué les stratégies d'alimentation fed-batch développées pour intensifier la production d'un vecteur viral dans des cultures de cellules HEK293. Tout d'abord, en utilisant les données de croissance et de métabolisme des cellules HEK293 accumulées dans la première partie de cette thèse, nous avons simplifié la stratégie d'alimentation dynamique développée en définissant un taux d'ajout spécifique par cellule (*cell specific feeding rate*, CSFR). Cette stratégie d'alimentation simplifiée a ensuite été appliquée à des cultures opérées en mode fed-batch et en mode hybride fed-batch/perfusion produisant un vecteur viral recombinant basé sur le virus de la stomatite vésiculaire (rVSV). Les cultures en mode hybride infectées entre $8\text{--}10 \times 10^6$ cellules/mL ont présenté une production totale presque 4 fois plus élevée et un rendement espace-temps 3.5 fois plus élevé par rapport à une culture référence en mode batch. Les cultures opérées en mode fed-batch ont permis de produire efficacement de rVSV dans des cultures infectées jusqu'à 8×10^6 cellules/mL, ce qui a permis d'augmenter de plus de 5 fois la production totale et

d'augmenter le rendement espace-temps d'un facteur 5.6 par rapport à la même culture référence en mode batch.

Dans l'ensemble, nous avons développé des stratégies génériques d'intensification capables de soutenir la production de virus influenza dans des processus à haute densité cellulaire. Bien qu'initialement établies dans des cellules HEK293 en suspension, les mêmes stratégies fed-batch ont été appliquées avec succès pour intensifier la production de virus influenza dans des cellules MDCK en suspension. Enfin, nous avons démontré la généralité des stratégies développées en les appliquant à la production d'un vecteur viral dans des cultures de cellules HEK293. Ce travail contribue à élargir l'arsenal de techniques de production à grande échelle de vaccins et de vecteurs viraux à partir de cultures cellulaires.

ABSTRACT

Cell culture processes are increasingly identified as a more flexible, scalable, and responsive alternative to traditional egg-based systems for the production of influenza virus vaccines. In order to become a cost-effective platform, cell culture-based virus production processes must be intensified, which up to now has been achieved almost exclusively through the development of perfusion operations. While perfusion processes typically support higher cell densities resulting in higher productivities, the large volumes of medium consumed raise challenges for medium preparation and handling, notably in large scale operations. In this scenario, the main goal of this doctorate research was to develop efficient feeding strategies to intensify influenza virus production in cell culture processes aiming to minimize medium consumption. To accomplish that, fed-batch, perfusion and hybrid feeding strategies were developed based on a streamlined approach for assessing the general metabolism of the cells based on key nutrients.

In the first part of this work, we developed processes to intensify the production of influenza virus in high cell density cultures of HEK293 cells. While using a shake tube scale-down model, we have designed a hybrid fed-batch/perfusion and a perfusion operation based on two different feeding strategies: i) a dynamic fed-batch strategy, in which addition of concentrated feed was performed based on the cell growth and the main metabolic profile of cells; ii) a perfusion strategy, in which the perfusion medium was supplemented with a concentrated feed to reduce the required perfusion rate (PR). The established processes were then scaled up to a benchtop bioreactor coupled to a Tangential Flow Depth Filtration (TFDF) membrane module as cell retention device. Successful influenza virus production was reported for cultures infected in the range of $8\text{--}10 \times 10^6$ cells/mL, resulting, for bioreactor runs, in more than 4-fold increase in total production and 3-fold increase in space time yield (STY) when compared to a low cell density batch reference. We have thus demonstrated that feeding strategies that limit medium consumption are suitable for large-scale influenza virus manufacturing.

In the second part of this thesis, we intensified the production of influenza virus in suspension MDCK cells, one of the preferred cell substrates for this virus. The main goal of this section was to evaluate if the strategies established for HEK293 cells could be applied for the production of the same virus using a different cell line. The dynamic feeding strategy developed was applied to hybrid fed-batch/perfusion and conventional fed-batch operations for influenza production in high

cell density cultures of MDCK cells. While fed-batch operations were affected by the cell density effect, with 20% reduction in the cell specific virus yield (CSVY) for cultures infected at 10×10^6 cells/mL, operation in hybrid mode supported successful virus production in cultures infected at up-to 15×10^6 cells/mL. When scaled up to a benchtop bioreactor coupled to a TFDF membrane module for cell retention, the hybrid process resulted in more than 8-fold increase in total production and 7.4-fold increase in space time yield when compared to a batch reference. Overall, we have demonstrated that hybrid processes constitute a valuable option for intensified influenza virus production in MDCK cells.

In the final part of this doctorate research, we evaluated if the strategies developed to produce influenza virus could be applied to intensify the production of other viruses. Over the last decades, a growing interest on the use of cell culture processes to produce viral vectors for vaccine and gene therapy applications have emerged. Therefore, we applied the developed fed-batch-based strategies to intensify the production of a viral vector in HEK293 cells. First, by using the growth and metabolic data for HEK293 cells accumulated in the first part of this thesis, we have simplified the developed dynamic feeding strategy through the definition of a cell specific feeding rate (CSFR). This simplified strategy was then applied to cultures operated in fed-batch and hybrid fed-batch/perfusion modes producing a recombinant viral vector based on the vesicular stomatitis virus (rVSV). Cultures infected between $8\text{--}10 \times 10^6$ cells/mL operated in hybrid mode presented almost 4-fold increase in total production and 3.5-fold increase in STY when compared to a batch reference. Operation in fed-batch mode allowed for successful rVSV production in cultures infected at up-to 8×10^6 cells/mL, resulting in more than 5-fold increase in total production and 5.6-fold increase in STY in comparison to the same batch reference.

Overall, we have developed generic intensified strategies capable of supporting influenza virus production in high cell density processes. Although initially established in suspension HEK293 cells, the same fed-batch-based feeding strategies were successfully applied to intensify the production of influenza virus in suspension MDCK cells. Finally, we have demonstrated the generality of the developed strategies by applying them to produce a viral vector in HEK293 cells. This work contributes to expanding the arsenal for large-scale cell culture-based production of viral vaccines and vectors.

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LISTE OF SYMBOLS AND ABBREVIATIONS

AS	Acoustic settler
ATF	Alternating tangential flow filtration
BR	Bioreactor
CDE	Cell density effect
CSVY	Cell specific virus yield
CSFR	Cell specific feeding rate
CSPR	Cell specific perfusion rate
DO	Dissolved oxygen
EMA	European medicines agency
FDA	Food and drug administration
GLN	Glutamine
GLU	Glutamate
GLUC	Glucose
HA	Hemagglutinin
HCD	High cell density
HIFB	High inoculum fed-batch
hpi	Hours post infection
IAV	Influenza A virus
IBV	Influenza B virus
IIV	Inactivated influenza vaccine
IS	Inclined settler
IVP	Infectious virus particles
LAC	Lactate

LAIV	Live attenuated influenza vaccine
MOI	Multiplicity of infection
MD	Medium dilution
MDCK	Madin-Darby canine kidney
ME	Medium exchange
NA	Neuraminidase
NH ₄	Ammonium
PR	Perfusion rate
q	Average cell specific consumption/production rate
QIV	Quadrivalent inactivated vaccine
RT	Residence time
RV	Reactor volume
SA	Sialic acid
STR	Stirred tank reactor
STY	Space time yield
TFF	Tangential flow filtration
TFDF	Tangential flow depth filtration
TIV	Trivalent inactivated vaccine
TMP	Transmembrane pressure
TOH	Time of harvest
TOI	Time of infection
TVP	Total volumetric productivity (same as VP)
VCD	Viable cell density
VP	Volumetric productivity (same as TVP)

VSV	Vesicular stomatitis virus
VVD	Vessel volume per day
WHO	World health organization
Y/M	Yield on media
Y_T	Total yield

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CHAPTER 1 INTRODUCTION

Influenza viruses represent an important burden for public health around the world, in the form of seasonal epidemics – which cause up to 650 000 deaths annually [1] – and, due to their high mutation rate, by posing the constant threat of a global pandemic [2-4]. Regular immunization campaigns with vaccines that match the current circulating strains is the most efficient preventive measure against the influenza disease, commonly known as “the flu” [5]. Although the majority of influenza vaccines is still manufactured using embryonated eggs [6], a cost-effective process established in the 1940s, cell culture processes are increasingly reported as a more responsive, flexible, and scalable alternative for vaccine production, notably during a potential pandemic scenario [7-10]. To this date, six cell culture-based influenza vaccines have been approved for use, but only three are still in production [8].

In order to become a more economically feasible option, cell culture-based influenza vaccine production processes must be intensified. This can be achieved by transitioning from conventional batch operations to more productive processes. A schematic representation of the modes of operation typically used for cell culture processes is presented in Figure 1.1. In batch operations, culture media and cells are added in the beginning of the process, with no further supplementation during the course of the run. Because of the fast depletion of nutrients and their finite supply, cell growth is limited to low cell concentrations. In fed-batch operations, addition of one or more nutrients in the form of a concentrated feed is performed during the run (continuously or intermittently) to prevent nutrient depletion [11]. As transformed mammalian cell lines present an inefficient metabolism, with high concentrations of key nutrients (glucose and glutamine) leading to increased production of toxic by-products (lactate and ammonium), efficient feeding strategies provide a constant nutrient supply while keeping them in low concentrations [12-17]. Because of the addition of nutrients, this type of operation supports cell growth to higher cell concentrations. However, due to the accumulation of toxic by-products (notably lactate and ammonium), cell growth is still limited. In perfusion operations, the bioreactor content is continuously removed and replenished with fresh media while cells are retained through a cell retention device. Due to the constant removal of toxic by-products and supply of fresh nutrients, this type of process supports cell growth to high cell densities in long culture durations [18-20]. While this type of process allows

for higher production in smaller footprints, the increased complexity and costs related to media consumption and handling require operations to meet a minimum productivity threshold [21, 22].

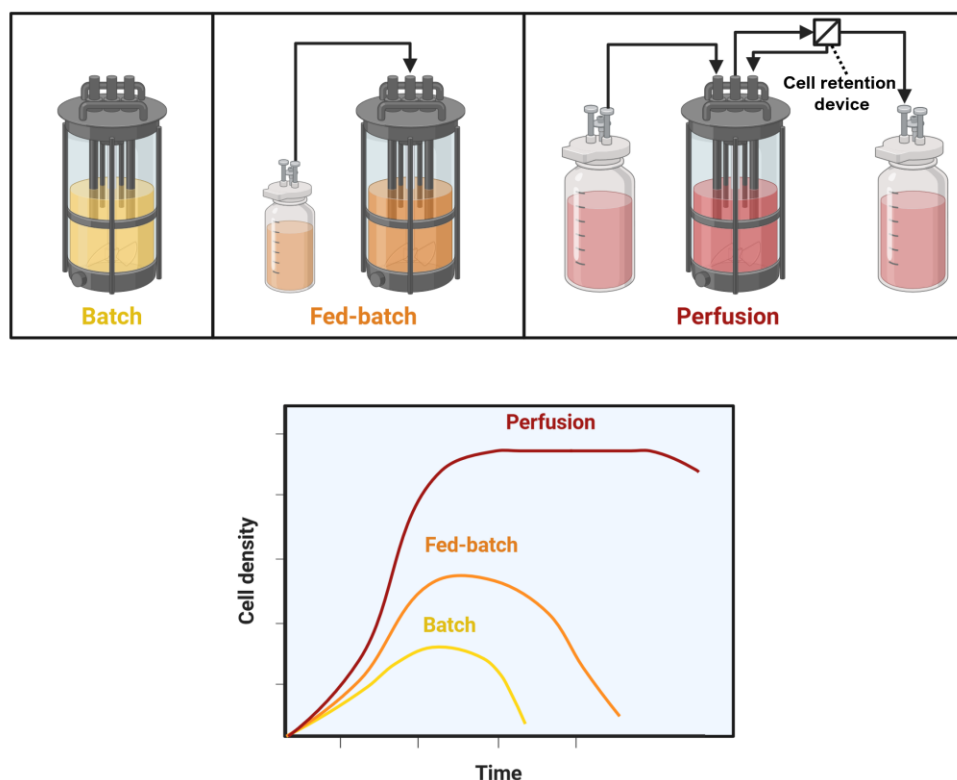


Figure 1.1 Schematic representation of modes of operation typically used for cell culture processes and their effect in cell density and culture duration. Created with BioRender.com and used under BioRender's Academic License terms.

While the intensification techniques developed for other cell culture processes – notably for recombinant protein production – can in theory be applied for virus manufacturing, the transient character of the latter as well as virus attributes such as size and instability demand specific attention. As infection processes typically lead to cell death, with infection phases lasting between 1–3 days, virus production can be augmented by increasing the cell density before infection. However, as nutrient and by-product concentration along with the metabolic state of cells have been shown to affect virus production at high cell densities [23, 24], an adequate feeding regime is required. Another challenge related to virus production processes is the sensitivity of the product

to conditions of the cell culture environment such as pH and toxic by-product concentration, notably lactate and ammonium [25]. One approach to alleviate that problem is by continuously harvesting the produced virus through a cell retention device, therefore reducing the average retention time of viral particles in the culture broth.

To sustain high cell densities, processes can be operated in perfusion and fed-batch. While perfusion processes typically achieve very high cell densities, thus increasing the productivity for a small footprint, the higher consumption of medium raises challenges with medium preparation, handling, and disposal, while increasing costs with consumables [18, 21]. Nevertheless, intensification of virus production processes has almost exclusively focused on perfusion operations [23, 26-34], as this type of process provides a constant supply of fresh nutrients and alleviate accumulation of by-products, which have been shown to be beneficial for virus production. Additionally, perfusion operations give the possibility for continuous harvesting the produced virus [35, 36]. Membrane-based cell retention devices are the preferred and most employed systems in the biopharmaceutical industry due to their scalability. However, membranes composed of different materials with various pore sizes were shown to be non-permeable to enveloped viruses [37], thus influenza [27, 34, 38], retaining most of the product in the bioreactor and hindering continuous harvesting approaches.

Fed-batch operations, on the other hand, are relatively simple, consume lower volumes of medium, and are the most well-established mode of operation in the biopharmaceutical industry [16, 39, 40]. However, as nutrients are constantly added in the form of a concentrated feed, the resulting accumulation of toxic by-products from cell metabolism limits the maximum cell concentrations achieved [21] and can negatively impact virus production. Nevertheless, new strategies for the design and control of feeding have been shown to sustain higher cell densities with limited by-product formation, and could be applied for virus production processes [16, 41]. As an alternative, hybrid processes, which combine fed-batch and perfusion operations [42-44], also have the potential to improve virus production by limiting medium consumption during the growth phase and alleviating by-product accumulation during the virus production phase.

Overall, while cell culture-based virus production processes have their particularities, they could certainly benefit from the knowledge and the techniques developed for the intensification of recombinant protein production, notably regarding fed-batch and hybrid operations.

In that scenario, the main objective of this work was to develop generic and scalable feeding strategies to intensify influenza virus production in cell culture processes, aiming to minimize medium consumption. To achieve that, fed-batch, low perfusion rate, and hybrid feeding strategies were developed based on the assessment of the general metabolism of the cells through a streamlined approach. The scalability of the developed processes was evaluated in benchtop bioreactors equipped with a membrane-based Tangential Flow Depth Filtration (TFDF) system as cell retention device. The permeability of the membrane to influenza viruses for the continuous harvesting of the product was assessed. While the processes were initially developed using HEK293 cells, they were later evaluated for influenza production in MDCK cells. Furthermore, the applicability of the process for the production of a different virus, produced likewise in HEK293 cells, was evaluated.

1.1 Presentation and organization of the document

This section introduces and describes the different parts of this document. Chapter 2 presents a literature review regarding the intensification of cell culture processes for influenza virus production. The latter advances and the research opportunities in the field are discussed in the form of a review article published in the Canadian Journal of Chemical Engineering, entitled: *Recent advances and current challenges in process intensification of cell culture-based influenza virus vaccine manufacturing*. Chapter 3 describes the research approach chosen for this thesis, as well as the research hypothesis and the objectives defined for this work.

In chapter 4, we describe the development of feeding strategies to intensify the production of influenza virus in HEK293 cells while minimizing medium consumption. This work is the subject of a research article published in Vaccines, entitled: *Intensified influenza virus production in suspension HEK293SF cell cultures operated in fed-batch or perfusion with continuous harvest*. In chapter 5 we apply the developed strategies to intensify influenza virus production using a different cell substrate. This work is presented in the form of a research article submitted for publication to the Journal of Biotechnology, entitled: *Fed-batch and perfusion strategies for process intensification of MDCK cell-based influenza vaccine production*. In chapter 6, we demonstrate the generality of the developed feeding strategies by applying them to intensify the production of a different virus using HEK293 cells. This work is the subject of a research article

submitted for publication to Biotechnology Progress, entitled: *Fed-batch strategies for intensified rVSV vector production in high cell density cultures of suspension HEK293 cells*.

Chapter 7 comprises a general discussion of the results obtained in this doctoral research, relating the outcomes to the previously defined research objectives. Finally, chapter 8 depicts the conclusions of this research as well as the recommendations for future work.

CHAPTER 2 LITERATURE REVIEW – ARTICLE 1 : RECENT ADVANCES AND CURRENT CHALLENGES IN PROCESS INTENSIFICATION OF CELL CULTURE-BASED INFLUENZA VIRUS VACCINE MANUFACTURING

2.1 Article presentation

The article presented in this chapter was published on April 16, 2021 in the Canadian Journal of Chemical Engineering. First, this literature review article depicts the manufacturing of cell culture-based influenza vaccines as well as its advantages and challenges when compared to the traditional egg-based production system. Then, the strategies used for the intensification of cell culture-based influenza virus production processes are detailed, and a critical analysis of the available literature is presented. Additional information, consisting of a schematic representation of an influenza A virus particle, is presented in section 2.8.

2.2 Recent Advances and Current Challenges in Process Intensification of Cell Culture-Based Influenza Virus Vaccine Manufacturing

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2.3 Abstract

Every year, millions of people are infected by the influenza virus around the world, which results in more than half a million deaths, particularly amongst the more vulnerable population. Since vaccination is the most efficient method of protection, millions of doses must be produced in a short period of time to supply seasonal vaccination campaigns around the globe, and billions of doses would be required to respond to a potential global influenza pandemic. The lack of flexibility of the current egg-based production system and its long production cycles have pushed biomanufacturers to invest in more flexible alternatives for vaccine production, particularly cell culture-based processes. While a valuable alternative, virus yields are still low, requiring extensive efforts to increase process productivity. Major efforts in the intensification of cell culture-based

viral vaccine manufacturing focus on the development of high cell density processes, which mostly involve the employment of perfusion-based strategies. In this review, some of the advantages of cell culture-based production of influenza vaccines will be discussed, and some of the current challenges and opportunities for the intensification of these processes will be presented. Finally, the recent advances in high cell density processes for influenza vaccine manufacturing will be reviewed.

Keywords : high cell density, influenza vaccine, perfusion, process intensification, suspension cells

2.4 Introduction

The influenza virus, pathogen that causes the influenza disease commonly known as the flu is responsible for worldwide seasonal epidemics and occasional pandemics. In the 20th century, three great influenza pandemics affected humanity as a whole: the Spanish flu, that is estimated to have killed between 20-50 million people from 1918-1920; the Asian flu, that killed up to 1.5 million between 1957-1958; and the Hong Kong flu, responsible for the death of 1 million people between 1968-1969 [3]. More recently, the swine flu, a milder but still important pandemic, is estimated to have killed 75 000 people in the US from 2009-2018 [45]. Even though the mortality rates of these three recent pandemics were much lower than those observed for the Spanish flu, our current experience with the ongoing COVID-19 pandemic raises concerns for the possible impacts of a new global influenza pandemic and stresses the need to tackle the challenges that still exist for large scale and rapid viral vaccine manufacturing. Additionally, every year, between 290 000-645 000 seasonal influenza associated deaths occur worldwide, especially among people older than 65 and children younger than 5 years [1].

2.4.1 Influenza Viruses

Influenza viruses are negative-stranded, segmented, and enveloped RNA viruses, and members of the Orthomyxoviridae family. Wild virions are pleomorphic, acquiring a roughly spheroidal shape after multiple passages, approximately 100 nm in diameter. The viral envelope consists of a host cell-derived lipid bilayer that contains, on the outside, three different transmembrane proteins, NA (neuraminidase), HA (hemagglutinin), and M2 (ion channel). HA, the most prevalent protein on the virus surface (approximately 80%), is a homotrimer in the form of a spike responsible for the initial attachment of the virus to host cell receptors with a terminal sialic acid (SA) and later

integration of the virus envelope to the cell membrane. NA, a homotetramer that represents around 17% of viral surface proteins, cleaves the SA from the host cell receptors, releasing viral progeny from the cell surface and resulting in further spread of the virus [46, 47]. Immunological response to influenza viruses usually results in the production of neutralizing antibodies against these two surface proteins, which prevents the infection of host cells [48].

Two types of antigenically distinct viruses, influenza A and influenza B, are responsible for the worldwide recurrent influenza outbreaks. Though influenza B virus (IBV) affects humans almost exclusively, influenza A virus (IAV) also infects a wide range of mammals and aquatic birds, having a greater impact on global health due to its greater genetic diversity – 18 HA and 11 NA subtypes were identified for IAV while only 1 type with two antigenic lineages has been defined for IBV [49]. Different HA subtypes are specific to different SA residues in the cell receptor, being a critical determinant for species restriction of IAVs and IBVs (avian influenza viruses have HAs specific to α 2-3 SA while human influenza viruses are specific to α 2-6 SA) [46, 48]. Frequent mutations on the antigenic portions of HA and NA proteins (antigenic drift) and genetic reassortments involving HA and NA encoding genes due to coinfection of a host cell with two different virus strains (antigenic shift) allow the influenza virus to escape pre-existing immunity or, more concerning, allow the virus to jump from one species to another, eventually infecting humans [3, 50]. The possibility of host adaptation due to the high mutation rate of the virus, the large reservoir of new influenza viruses in different animal species, particularly aquatic birds, and constant animal-human contact due to animal farming, are all factors that contribute to the increasing risk of a new global pandemic.

2.4.2 Influenza Virus Vaccines

A vaccine is a biological preparation containing immunogenic material of a specific pathogen (attenuated, inactivated agent, recombinant vectored, or subunit) that can generate immunity against this infectious agent. Three types of vaccines against influenza are available on the market: inactivated influenza vaccine (IIV) (whole virus, split virus, and subunit), live attenuated influenza vaccine (LAIV), and recombinant vaccine (RV). Among IIVs, which represent the majority of currently approved vaccines, the trivalent inactivated vaccine (TIV) contains immunogenic material from two IAV and one IBV strains, dosed according to its HA content (15 μ g for each strain) [7, 48]. Quadrivalent vaccines (QIVs), which include an additional IBV strain, are

increasingly being developed so as to improve vaccine efficacy. Four LAIVs are commercially available: FluMist, (quadrivalent, AstraZeneca PLC), Ultravac (trivalent, Microgen), Nasovac (trivalent, Serum Institute of India Pvt. Ltd) and one trivalent LAIV (no commercial name, Changchun BCHO Biotechnology Co). The vaccines, which contain the attenuated virus adapted for growth in lower temperatures, are intranasally administered and have shown to be efficacious in young children [51]. FluBlok (Sanofi-Pasteur) is the first RV for influenza approved by the FDA. It contains recombinant HA protein from different strains, produced in insect cells (45 µg for each strain, quadrivalent) [52, 53].

2.4.3 Timeline and Logistics for Influenza Vaccine Manufacturing

Because of the high mutation rate of this virus, a constant surveillance of influenza activity must be done in order to assure a good match between the circulating strains and the vaccine produced for the season. For this reason, the World Health Organization (WHO), together with more than 100 national centres, performs the monitoring, isolation, and identification of influenza viruses causing localized epidemics all over the world throughout the year [54]. From the data generated, the WHO selects and announces the four influenza strains that are likely to circulate for the next flu season. From the time of this announcement, which is made in February for the Northern Hemisphere and in September for the Southern Hemisphere, manufacturers have approximately 6 months to produce and supply vaccines for the vaccination campaigns that start in September and April in the Northern and Southern Hemispheres, respectively. For both LAIV and IIV, 84.5% of currently licenced vaccines are still produced using embryonated hen's eggs as at 2019 [55], a method established in 1940 where fertilized eggs are used for replication of the virus [7]. Various factors may challenge the production deadline: absence of the specific reagents for influenza quantification, fulfillment of regulatory aspects, generation of high growth reassortants adapted to egg-growth (especially for H3N2 and IBV strains), continuous egg supply, and full characterization of the product [7, 51].

Despite WHO's growing efforts in increasing global production capacity in response to the threat of a new global pandemic, namely through the Global Action Plan for Influenza Vaccines (GAP), a comprehensive strategy developed between 2006-2016 which targeted a global production sufficient to immunize 70% of the population within a six-month period, current manufacturing capacity is still not sufficient. According to Sparrow, et al. [55], around 8.31 billion doses of

pandemic vaccine could be produced in a 12-month period with the current manufacturing capacity. While this number may seem high at first glance, this estimation is made based on several assumptions, such as an adequate supply of eggs and an immediate start in production, which might not be the case if manufacturers are in the middle of the manufacturing process for seasonal influenza vaccines [55]. Additionally, more than one dose may be required in order for the new pandemic vaccine to be efficient and pandemic strains other than H1N1, such as H5N1, have already shown lower yields in eggs [56].

2.4.4 Cell Culture-Based Influenza Vaccines

Over the last few decades, mammalian cell culture has been increasingly used by the biopharmaceutical industry for recombinant protein production [57-59]. Major advances in cell culture techniques, such as the possibility of operating with high cell densities in strictly controlled bioreactors with volumes of 10 000 L or larger [60], have drawn the attention of influenza vaccine manufacturers into investing in the development of cell culture-based vaccine production as a faster alternative to the conventional egg-based technology. Cell culture-based viral vaccine production includes the production of whole viruses for inactivated vaccines using avian or mammalian cells, as well as the production of viral proteins for recombinant vaccines, performed mostly using insect cells. Here, we will be discussing exclusively the first case. Briefly, cells are cultivated until sufficient cell density is achieved (growth phase), followed by the infection and replication of the desired virus strain (infection phase). Advantages of cell culture-based production over traditional egg-based technology include: (i) scalability, especially when using suspension growth cells [20]; (ii) greater process control that allows a more reliable and well characterized product [61]; (iii) more similarity between vaccine composition and circulating strains, since egg growth adaptation might generate unwanted antigenic modifications [62, 63]; and (iv) a faster response in the case of a pandemic due to short production cycles, since cell-based production can be accelerated by the use of frozen high cell density seed stocks, in contrast with egg supply, which requires longer timelines [7, 51, 54, 64]. In fact, in a recent study performed by Sparrow, et al. [55] aiming to assess global production capacity for seasonal and pandemic influenza vaccine, egg supply was shown to be one of the production bottlenecks for a considerable fraction of active vaccine manufacturers.

Cell cultured-based production of influenza viruses has gone through major advances over the last decade. Currently, six vaccines produced using cell culture systems are approved for production around the world (Table 2.1). However, several challenges still have to be overcome in order to further encourage manufacturers to shift from traditional cost-efficient egg-based production to cell culture-based processes. This review will present some of the current challenges for large-scale production of cell culture-based influenza vaccines as well as the recent advances in the intensification of its production process.

Table 2.1 Licensed cell culture-based influenza vaccines

Commercial name	Manufacturer	Composition	Cell platform	Approval	Current production status
Preflucel	Baxter	trivalent	Vero	EU	No longer in production
Celvapan	Baxter	monovalent (H1N1)	Vero	EU	No longer in production
Flucelvax	Seqirus	trivalent and quadrivalent	MDCK	FDA/EU	In production
Audenz	Seqirus	monovalent (H5N1)	MDCK	FDA	Not in production
Flublok	Sanofi Pasteur	trivalent and quadrivalent	Insect cells (derived from Sf9)	FDA	In production
SKYCellflu	SK Bioscience	quadrivalent	MDCK	MFDS	In production

2.5 Current Challenges and Opportunities in Process Development of Cell Culture-Based Influenza Vaccine Manufacture

2.5.1 Adherent and Suspension Host Cell Lines

Successful production of influenza virus was reported for different cell lines, including Madin Darby canine kidney cells (MDCK) [65-70], adherent monkey kidney cells (Vero) [70-72], suspension human embryonic retinal cells (PER.C6) [73], suspension Muscovy duck cells (AGE1.CR and AGE1.CR.pIX) [26, 29, 74-76], and suspension human embryo kidney cells (HEK-293) [25, 77, 78]. So far, MDCK cells have been the preferred cell line for influenza vaccine production, offering the advantages of being resistant to different growth conditions, being susceptible to a limited number of mammalian viruses [79] (therefore acting like a filter for adventitious agents), and reporting virus yields comparable to the ones obtained in the egg-based process [66, 68]. There are, to this date, three influenza vaccines produced using MDCK approved for use (Table 2.1), Flucelvax (Seqirus), Audenz (Seqirus), and SKYCellflu (SK Bioscience), produced using MDCK cells adapted to suspension growth. Although originally adherent, MDCK

cells adapted to suspension growth have exhibited comparable or higher virus production than non-adapted cells [68]. In fact, cells growing in suspension are much more attractive from an industrial point of view as the scale up is facilitated (requiring an increase in volume in contrast to increase in area for adherent cell cultures), and one can more easily achieve high cell densities in this type of process, allowing the use of more compact bioreactors and the achievement of higher volumetric productivities without nutrient or oxygen limitations [20, 80]. However, adaptation of cells to suspension growth has been shown to increase tumorigenicity for different cell lines [81], including MDCK cells [82, 83]. Kluge, et al. [82] performed a proteomic monitoring of three MDCK cell lines independently adapted to suspension growth and reported an increase in the levels of proteins related to carcinogenesis, highlighting the need of proteomic studies for the detection of tumorigenicity markers in early stages of cell line development. Vero cells, one of the most accepted continuous cell lines for the production of human vaccines [84], have also been employed for commercial production of cell culture-based influenza vaccine. The extensive experience gathered by the use of this cell line since its approval by the WHO in 1987 [85] has provided considerable evidence to support its safety of use. Two cell-based influenza vaccines produced using this system, Preflucel and Celvapan (Baxter), which are no longer in production, have been licensed in the EU. Although these cells can be adapted to suspension growth [86, 87], current virus production using this system relies almost exclusively on adherent cell culture [88], performed in large scale through the use of microcarriers – small beads designed to remain in suspension in agitated tanks on which adhered cells grow – a technology that, although efficient, remains limited by the surface area available for cell growth.

The screening and improvement of new host cell lines is critical for the development of cell culture-based vaccine manufacture. Characteristics such as suspension growth and the ability to grow in the absence of animal-based supplements are preferable since scale up and product quality control are some of the main challenges for cell culture-based IAV production. It is worth noticing that, in order to be used for human vaccine production, continuous cell lines must be assessed for their safety, and their use must be approved by specific regulatory bodies. These assessments include testing for the presence of endogenous and adventitious agents and evaluating cell's oncogenicity and tumourigenicity as well as the stability of the cell substrate [89].

2.5.2 Other Influenza Subtypes

While almost every study on cell culture-based influenza virus production was done using the H1N1 subtype, more specifically with the A/PR/8/34 strain, IAV vaccine manufacture involves the production of different IAV subtypes, such as the seasonal H3N2 and the potential pandemic candidates H7N9 and H5N1. Because these subtypes usually present a poor yield when produced in eggs, vaccine manufacture currently relies on the production of high growth reassortants (HGRs), a modified virus that expresses the HA and NA proteins from the desired strain and all the other genes from a high growth strain such as the A/PR/8/34 H1N1 [7]. This is done by co-infecting the same egg with both strains and selecting the reassortant with the desired characteristics. This process, performed by only a few laboratories in the world, takes from 4-6 weeks to be completed [55], time that could be critical in the case of a pandemic. Although it is still not clear if this process would be required for cell culture-based IAV production, some evidence suggests that HGRs do not show improvement in yield in cell culture systems when compared to the wild type virus [90].

The H5Nx IAV subtype is one of the potential candidates for a global influenza pandemic. Although this avian virus has already caused some isolated infections in humans with a 60% mortality rate, its main host species remains the poultry. However, the risk of host adaptation from the accumulation of adaptive mutations remains [56, 91]. Given the increased pathogenicity of H5Nx strains, which usually results in the death of the infected egg, its production using the traditional egg system relies on the generation of a less pathogenic virus by a highly complex process involving reverse genetics [56]. Cell culture-based systems have shown to be a good alternative for the production of this pandemic subtype with three active influenza vaccine manufacturers currently producing vaccines for H5N1 using this system [55].

After its appearance in 1968 caused a mild pandemic, the H3N2 IAV subtype has now circulated for over 50 years, causing seasonal endemics around the globe and being present in all trivalent and quadrivalent seasonal IIVs. Evidence shows that the egg-adapted version of the strain used for the 2016-2017 seasonal vaccine production lacked one important glycosylation site, which is one of the most probable causes for the low estimate vaccine effectiveness of only 43% [63]. The use of cell culture for both isolation and production of this IAV subtype could represent an increase in vaccine effectiveness since egg growth adaptation and HGR production would not be necessary.

Cell culture-based H3N2 IAV production has been evaluated using adherent MDCK and Vero cells [92].

The avian H7N9 IAV subtype, another potential threat for a global pandemic, has already caused isolated human infections with a high mortality rate of >25% [93]. As for H5N1, while its main host species is the poultry, accumulated mutations could result in a human-adapted strain for which humans have no protective immunity. To the best of the authors' knowledge, no data is available for the production of the potentially pandemic H7N9 subtype using the cell culture system.

Although IBVs are not known to cause pandemics, the high burden of these viruses in seasonal epidemics requires its addition to all seasonal trivalent and quadrivalent vaccines [91]. Though data on cell culture-based production of IBVs are scarce, these viruses have shown good growth in MDCK cells [71, 92].

2.6 Advances in Process Intensification of Cell Culture-Based Influenza Vaccine Manufacture

The first definitions of process intensification appeared in the second half of the 90s [94], describing it as an approach to decrease the size of the equipment in a chemical plant or decrease the number of steps in a process while achieving the same production goal. A broader definition came later on, with Stankiewicz and Moulijn [95], who defined process intensification as not only a decrease in the overall plant size, but also a dramatic increase in productivity, a reduction in the consumption of energy, or smaller waste and by-product formation. However, the discussion over what defines a given improvement as important enough varies according to the field of study. When applied to cell culture-based viral vaccine production, the main focus of process intensification is the improvement of virus titres and process productivity. In order to improve virus titres, three critical factors should be considered [80]:

- Cell density and metabolic state of the cells: Usually, virus titres are strongly related to the cell density at infection. Virus production is divided in a cell proliferation phase followed by a replication phase, where complex mechanisms related to the viral infection take place (virus entry in the cell, viral protein synthesis, budding, and progeny release) [47, 96]. Since cells usually cease to divide after infection, high cell densities must be achieved during the growth phase and infection is normally performed at the late exponential growth phase [25, 28, 29]. However, a drop in cell specific productivity (viral particles per cell) has been

observed for cultures infected in high cell densities, which has been attributed to nutrient limitations and by-product accumulation [23].

- Virus concentration at the time of infection, that is, the number of infectious virus particles per cell or multiplicity of infection (MOI): Although different infection cycles occur during the replication phase, final titres are greatly related to the initial concentration of virus used. While a high enough MOI should be applied in order to assure that a significant number of cells will be synchronously infected in the first infection cycle, a too high MOI may lead to the activation of apoptotic pathways and premature cell death [77]. A MOI between 10^{-1} and 10^{-5} (IVP/cell) is usually employed for cell culture-based influenza virus production.
- Viral particles residence time in the reactor and culture duration: In order to minimize viral particle degradation, it is critically important to limit its residence time (RT) in the reactor. When product is retained inside the reactor, as in batch, fed-batch, and concentrated-fed-batch operations, the RT is equal to the culture duration. In this case, the moment when the product is harvested, namely time of harvest, must be carefully determined in order to not only minimize degradation but also to limit protein and DNA contamination due to cellular lysis. As an alternative, continuous processes offer the advantage of a constant product harvest and a small viral particle RT. However, disadvantages of this type of process include an increased risk of contamination due to longer production cycles and product dilution, which increase costs related to further downstream processing. In fact, as product dilution and concentration of contaminants are determinant for downstream processes' efficacy, one should keep these in mind when intensifying virus production processes. Additionally, advances in integrated continuous processing, notably for recombinant protein production [61, 97, 98], could eventually be adapted for semi-continuous integrated virus manufacturing.

In order to reach high cell concentrations, HCD processes normally make use of fed-batch and/or perfusion strategies to keep nutrient levels above minimum thresholds. Fed-batch processes are the current standard in the pharmaceutical industry, owing to their relative operational simplicity and their increased productivity. Feeding is performed by the addition of concentrated media, and the volume to be added is often determined as to keep nutrient concentration, usually glucose, inside a specific range [11]. Although fed-batch operations alleviate nutrient limitations, accumulation of

toxic by-products, such as ammonia and lactate, limits maximum cell density that can be achieved [21].

In perfusion cultures, on the other hand, part of the bioreactor content is removed and replenished, at the same rate, with fresh media, while cells are kept inside the reactor by a cell retention device. This allows not only for the maintenance of a certain nutrient level but also the constant removal of toxic by-products [18]. As a result, much greater cell densities (more than 70×10^6 cell/mL for perfusion cultures compared to 20×10^6 cell/mL for fed-batch and up to 6×10^6 cell/mL for batch) can be achieved, leading to higher productivities and therefore allowing the utilization of smaller bioreactors for the same final yield [21]. However, increased cost of goods, especially related to increased fresh media consumption, and greater complexity associated with fluid handling and cell retention, are the major drawbacks related to this type of operation [22]. In perfusion cultures, the rate of media exchange can be described by the perfusion rate (PR), expressed in vessel volumes of media (volume of media per bioreactor volume) per day (vvd^{-1}), or by the cell specific perfusion rate (CSPR), expressed by the volume of media exchanged per cell per day ($\text{pL} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$). While the perfusion rate represents an operational volume, the CSPR can be used to control media exchange based on cell growth. This is done by adjusting the perfusion rate according to cell concentration so as to maintain a fixed CSPR, that is, a fixed media exchange rate per cell [18]. In a simpler set up, cell density is assessed off-line at given intervals, and perfusion rate is manually adjusted to keep a constant CSPR. A tighter control can be achieved by monitoring cell concentration in real time and automatically adjusting PR. Cell specific perfusion rate is usually set between $10\text{-}60 \text{ pL} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ [20, 26, 28, 29, 99]. A number of methods such as microscopic imaging, optical sensors, and conductivity and capacitance sensors were already applied for cell density monitoring with the latter being the most commonly employed [16].

In order to evaluate and compare virus production processes, two parameters are normally calculated: total volumetric productivity (TVP) and cell specific virus yield (CSVY). TVP takes into consideration the total volume of spent media, the duration of the culture and the total yield of virus. This parameter is especially useful to compare perfusion cultures, which usually consume high amounts of fresh media, with fed-batch and batch processes. CSVY represents the average amount of virus produced by a single cell and it is calculated by dividing the total virus yield by the total amount of cells in the reactor. It is a critical parameter to evaluate the performance of virus production processes since the metabolic state of cells has an important effect on virus yields. It is

worth noting that comparisons between CSVY values should be done with caution since different studies employ different cell concentrations for the evaluation of this parameter (cell concentration at the time of infection, maximum cell concentration post-infection, cell concentration at harvest).

As mentioned before, virus production processes are normally divided in two phases: first, cells are allowed to proliferate until a high cell density is achieved, followed by virus infection, usually performed at the late exponential phase of growth. Cell density at infection and metabolic state of cells during infection play critical roles in final virus titres and therefore, a constant supply of nutrients and limited accumulation of toxic by-products are essential for achieving high yields and to minimize the cell density effect, the latter being characterized by a reduction in CSVY when infection is performed at high cell concentrations. Perfusion-based strategies address both of these problems and are usually the choice for virus production intensification. Table 2.2 summarizes high-cell-density influenza virus production processes using suspension cells and its main operation parameters. Since CSVY values are highly dependent on the cell line employed for virus production, comparisons between different processes should be done with caution and preferably be done amidst processes employing the same cell system.

In regard to the cell retention device, membrane-based filtration systems (alternating tangential flow filtration [ATF] and tangential flow filtration [TFF]) and acoustic filters are most commonly employed for virus production (Table 2.2). While membrane-based filters offer the possibility of retaining or harvesting viruses from the bioreactor, depending on pore size [37], membrane clogging and increased shear stress, especially for TFF systems, are the main drawbacks related to this type of device. Nikolay, et al. [28] evaluated TFF and ATF systems for a high-cell-density production of yellow fever virus and obtained virus titres 20-fold lower for the TFF system when compared to the ATF, which was attributed to increased shear stress in the module. Membrane clogging and unspecific product binding may also result in an inefficient virus harvest, even for membranes with larger pore sizes. Genzel, et al. [27] and Vazquez-Ramirez, et al. [29] reported the retention of most of the viral particles inside the reactor for membrane pores $< 0.5 \mu\text{m}$, as well as product losses by membrane entrapment of cellular debris and viral particles. Acoustic settlers (AS) and inclined settlers (IS), on the other hand, offer the advantage of continuous virus harvest and reduced shear stress, which can be critical if perfusion is to be maintained during the infection phase since infected cells tend to be more fragile [23]. Granicher, et al. [26] and Coronel, et al. [76] evaluated a full perfusion process with continuous virus harvest for IAV production using an IS

and an AS, respectively, as cell retentions devices. Parameters such as temperature of the heat exchanger and recirculation strategies were shown to have a great impact over process productivity.

Perfusion processes using HEK-293 cells for the production of IAV, operating with a fixed PR and using an AS as cell retention device, have been reported [25, 78]. When evaluating IAV production in HEK-293 cells, Petiot, et al. [25] obtained maximum cell densities of 15×10^6 cells·mL⁻¹ when operating at a fixed PR of 0.5 vvd⁻¹, resulting in a 10-fold increase in total virus yield and higher CSVY when compared to batch. The effect of temperature in virus stability was reported; while different temperatures of production and storage did not significantly alter the HA content, virus production at 35°C with continuous harvest and storage at 4°C reduced the degradation of infectious particles by 1-log, a reduction that could be critical for LAIV production.

IAV production in high-cell-density perfusion processes employing an ATF system as cell retention device has also been reported. Genzel, et al. [27] evaluated the HCD production process of IAV using human (CAP) and duck (AGE1.CR) suspension cell lines. PR was manually adjusted so as to maintain nutrient levels above a minimum set point. CSVYs comparable to batch culture were obtained for both cell lines, with maximum cell densities of 32 and 52×10^6 cells·mL⁻¹, respectively. However, high media consumption in perfusion cultures resulted in TVPs 2-fold lower than those obtained for batch culture. A reduction in media consumption was reported (by Vazquez-Ramirez, et al. [29], when producing IAV in AGE1.CR.pIX cells, directly derived from the AGE1.CR cell line, when a CSPR-based hybrid fed-batch/perfusion strategy was employed. Bioreactors were operated in perfusion during the cell proliferation phase, shifted to intermittent fed-batch until 24 hpi (hours post infection) followed by a pH-controlled perfusion rate until the end of the culture. Two-fold higher virus titres and CSVY and TVP comparable to batch were reported for the hybrid strategy. Other CSPR-based perfusion strategies for IAV production employing the same cell line and different cell retention devices are reported [26, 76]. When evaluating infections at very high cell densities (25×10^6 cells·mL⁻¹) and different recirculation strategies for an inclined settler as cell retention device, Coronel, et al. [76] obtained CSVYs and TVPs up to 4.7-fold and 2.2-fold higher, respectively, than a control run employing an ATF system. The lower productivity obtained with the membrane-based module is believed to be related to a higher shear stress as well as with a non-efficient virus harvest due to membrane clogging, resulting in virus degradation inside the reactor. Granicher, et al. [26] evaluated different recirculation strategies (pump-based and valve-based recirculation) for an AS to evaluate perfusion-based IAV

production using the same cell line (AGE1.CR.pIX). CSVYs and TVPs up to 1.5-fold higher were obtained for this system when compared to an ATF control run. The temperature range in the inlet and outlet of the AS, the recirculation strategy, and the recirculation rate were shown to have a significant impact over final virus titres. Measured shear rates obtained for the AS were up to 100-fold lower than in the ATF module, confirming the hypothesis that higher shear stress in the ATF system contributes to the lower virus titres obtained.

Table 2.2 Overview of high-cell-density processes for virus production using suspension cells.

Virus	Cell line	Bioreactor type/volume	Proliferation phase	Infection phase	Media exchange	Cell retention device	Maximum cell density	Comments	Ref.
Influenza A H1N1	HEK 293 (human)	STR / 3.5 L	Perfusion (Fixed PR 0.5 vvd ⁻¹)	Perfusion (Fixed PR 0.5 vvd ⁻¹)	No	Acoustic settler	15 (10 ⁶ cells.mL ⁻¹)	- Yield on media comparable to or higher than that of batch culture. CSVY two-fold higher than that of batch culture. Considerable effect of temperature on virus stability (temperature shift to 35°C and virus storage at 2-8°C)	[25]
Influenza A H1N1	AGE1.CR (duck)	STR/ 1L	Perfusion (Manual control of PR)	Perfusion (Manual control of PR)	No	ATF	52 (10 ⁶ cells.mL ⁻¹)	- Comparable CSVY ^a but 2-fold lower TVP ^b when compared to batch cultures. Considerable effect of membrane pore size on virus production.	[27]
Influenza A H1N1	CAP (human)	STR/ 1L	Perfusion (Manual control of PR)	Perfusion (Manual control of PR)	No	ATF	32 (10 ⁶ cells.mL ⁻¹)	- CSVY ^a and TVP ^b comparable or higher than that of batch culture. Considerable effect of membrane pore size on virus production.	[27]
Influenza A H1N1	AGE1.CR.p IX (duck)	STR/ 1L	Perfusion (CSPR-based manual control of PR)	Intermittent fed-batch / perfusion (pH-based control)	Yes	ATF	23 (10 ⁹ cells.mL ⁻¹)	- Concentration step before infection. CSVY ^a and TVP ^b comparable or slightly lower than that of batch culture and four-fold and three-fold higher, respectively, when compared to full perfusion. pH perfusion control led to 4-fold higher media consumption.	[29]
Influenza A H1N1	AGE1.CR.p IX (duck)	STR/1L	Perfusion (CSPR-based manual control of PR)	Perfusion (Fixed PR of 2 vvd ⁻¹)	No	Inclined settler/Acoustic settler/ATF	50 (10 ⁶ cells.mL ⁻¹)	- Infection at very high cell densities (25x10 ⁶ cells.mL ⁻¹) using IS as retention device resulted in CSVYs and TVPs up to 4.7-fold and 2.2-fold higher, respectively, than control run employing an ATF system. CSVY up to 2.6-fold higher than batch runs using the same cell line. The recirculation strategy was shown to have a great impact over productivity. The lower temperature on the heat exchanger seemed to increase virus titers due to increased stability. Infectious titers were similar for all of the retention devices employed.	[76]
Influenza A H1N1	AGE1.CR.p IX (duck)	STR/1L	Perfusion (CSPR-based manual control of PR)	Perfusion (Fixed PR of 1.5 or 2 vvd ⁻¹)	Yes	Acoustic settler/ ATF	70 (10 ⁶ cells.mL ⁻¹)	- Infection at very high cell densities (25x10 ⁶ cells.mL ⁻¹) using AS as retention device resulted in CSVYs and TVPs up 1.5-fold higher than control run employing an ATF system. The recirculation strategy was shown to have a great impact over productivity. Infectious titers were similar for all of the retention devices employed. Measured shear rate was reported to be up to 100-fold higher in the ATF module when compared to the AS.	[26]

^a CSVY = cell-specific virus yield [virions.cell⁻¹ ; PFU.cell⁻¹ ; VP.cell⁻¹]

^b TVP = total volumetric productivity, considering the total volume of media, maximum virus yield and time to achieve this yield [virions.mL⁻¹.day⁻¹; VP.mL⁻¹.day⁻¹; PFU.mL⁻¹.day⁻¹]

2.7 Conclusions and Future Perspectives

As discussed, the traditional egg-based manufacture of influenza vaccines has a number of drawbacks, particularly its long production cycles, which could be critical in the case of a potential pandemic. It should be pointed out that emerging vaccine technologies, notably the use of mRNAs that gained ground during the COVID-19 pandemic, may revolutionize viral vaccine manufacturing. In fact, mRNA vaccines against influenza viruses have already started phase 1 clinical trials [100]. However, it is likely that different vaccine technologies will prove to be best suited for different groups of the human population and various production platforms will be required for worldwide supply. Cell culture-based processes could be part of the long-term solution for influenza vaccine manufacturing, as the development of large-scale industrial operations could certainly benefit from the technological advances pushed by recombinant protein production using mammalian cells. However, the high level of capital investment required to establish a cell culture-based vaccine facility, as well as to further develop upstream and downstream processes, has not traditionally encouraged influenza vaccine manufacturers to make the change. Additionally, almost all of the latest developments in the intensification of upstream processes for influenza virus production involve perfusion-based strategies. While this type of process can be more easily implemented for recombinant protein production, with very high cell densities and volumetric productivities in long and stable operations [19-21], the transient nature of influenza production processes represents one of the biggest challenges for large-scale cost-effective vaccine manufacture. Still, much is yet to be done for the intensification of cell culture-based influenza virus production. The evaluation of new hybrid production strategies, the development of better online monitoring techniques, the screening of suspension cell lines with improved characteristics, the evaluation of different cell retention devices, and the development of culture media and media supplementation are all examples of areas that could be further improved.

2.8 Additional information

A detailed schematic representation of an influenza A virus particle is presented in Figure 2.1. HA (haemagglutinin), NA (neuraminidase), M1 and M2 (matrix proteins), NS1 and NS2 (non-structural proteins), NP (nucleoprotein), PA, PB1, PB2 (viral replication proteins).

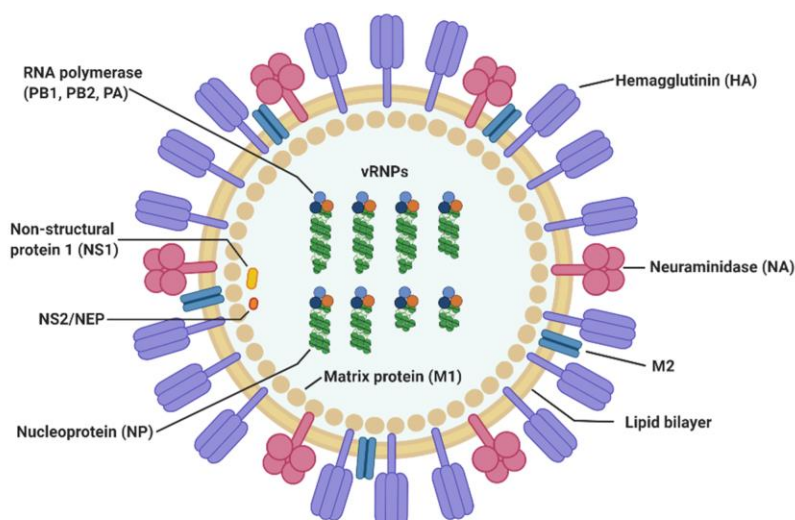


Figure 2.1 Schematic representation of an Influenza A virus particle. Figure from Jung and Lee [101], used under Creative Commons CC-BY license.

2.9 Key points

The presented article reviews cell culture-based influenza virus vaccines and its manufacturing, as well as the current challenges and opportunities for intensification of this type of process.

Influenza viruses are an important burden for public health systems around the world, causing seasonal epidemics and posing a constant threat of a global pandemic. While vaccines are the most efficient measure against this virus, manufacturing of seasonal vaccines and pandemic preparedness represent a huge logistical and technological challenge. Our recent experience with the COVID-19 pandemic has shown that the development of multiple platforms for vaccine manufacture might be critical to guarantee worldwide supply of vaccines.

When compared to the traditional egg-based vaccine production systems, cell culture processes present key advantages:

- Scalability, notably for processes employing suspension cell lines, as large-scale cell culture techniques have greatly advanced due to the recombinant protein industry.
- Fast response in case of pandemics, as cells can be stocked in HCD seed banks, resulting in shorter production cycles.

While the screening and improvement of suspension cell lines is critical, new cell substrates must be assessed and approved by regulatory bodies. The adaptation and use of well-established cell substrates can facilitate product approval.

Due to the transient nature of virus production processes, the strategies used for the intensification of this type of process differ from traditional recombinant protein production, focusing in two main factors:

- Increasing the cell density at time of infection, while maintaining cells in a desired metabolic state, to avoid the so called “cell density effect”. This is achieved with the development of feeding strategies, which may consist of:
 - Fed-batch: well-established operation with reduced complexity and costs; leads to the accumulation of toxic by-products that can decrease productivity.
 - Perfusion: typically allows for high productivities in small footprints; increases costs related to culture media, preparation and handling of liquids.
- Decreasing the retention time of virus particles in the reactor with continuous harvest strategies, to avoid product degradation. The selection of the cell retention device is critical to ensure product passage.
 - Membrane-based systems: scalable and well established in the industry; virus retention due to progressive membrane clogging has been reported for different materials and pore sizes.
 - Non-membrane-based systems: not available for large-scale manufacturing; allow for virus passage and continuous harvest.

Efforts for the intensification of influenza virus production have almost exclusively focused on the development of perfusion strategies which, although resulted in important increases in productivity, have also increased significantly process complexity and media consumption.

CHAPTER 3 GENERAL ORGANIZATION AND RESEARCH APPROACH

This section presents the research hypothesis, details the general objective formulated in chapter 1, and describes the research approach chosen for the project. In addition, the general organization of the work, which comprises four articles, is presented.

3.1 Research hypothesis

The general hypothesis of this research project was the following:

– Research hypothesis –

A generic and scalable process, designed based on fed-batch and/or low perfusion rate strategies, is capable of sustaining cell growth and intensified influenza virus production while minimizing media consumption in high cell density cultures, resulting in a significant increase in productivity when compared to low cell density batch cultivations.

Following this general proposition, two subsequent hypotheses were developed:

– Derived hypothesis n° 1 –

The developed process is able to sustain intensified influenza virus production in high cell density cultures of different suspension cell lines.

– Derived hypothesis n° 2 –

When applied to a same cell line, the developed process is able to sustain intensified production of other viruses.

3.2 Research objectives

The general objective of this research project, formulated based on the previously defined research hypotheses, was the following:

– Research objective –

Develop a generic and scalable process that minimizes medium consumption based on fed-batch and/or low perfusion rate strategies for intensified influenza virus production in high cell density mammalian cell cultures.

To achieve this objective, three specific objectives were defined:

– *Specific objective n° 1* –

Develop and scale-up a process that minimizes medium consumption for intensified influenza virus production in high cell density cultures of HEK293 cells.

Two different processes were designed and evaluated, using HEK293 cells as a model cell substrate and influenza (A/PR/8/34 H1N1) as model virus. To limit the consumption of medium, both processes were based on fed-batch and/or low perfusion rate feeding strategies. As high cell density cultures require a constant and adequate nutrient supply, and as viral particles are known to be sensitive to the cell culture environment, the evaluated processes included:

- A hybrid process, operated in fed-batch during the growth phase with a continuous harvest step (perfusion) after infection. To accommodate the metabolic variations that naturally occur during longer cell culture processes, a dynamic feeding strategy based on cell specific nutrient consumption was chosen for the fed-batch step.
- A perfusion operation with a low perfusion rate (limited to 0.5 VVD). To ensure the supply of non-quantified nutrients, the perfusion medium was supplemented with concentrated feed medium.

This work is the subject of chapter 4, presented in the form of an article published in *Vaccines* in 2023.

– *Specific objective n° 2* –

Evaluate the developed process for intensified influenza virus production in high cell density cultures of MDCK cells.

While we have chosen the HEK293 cell line as a model for this work, a generic process should be applicable to different cell lines while requiring only minor rational changes to accommodate differences in metabolism. Therefore, we have chosen to validate this premise by evaluating one of the developed processes for the production of influenza virus in high cell density cultures of suspension MDCK cells. As the characteristics of growth and nutrient consumption varied between the two cell lines (growth rate, maximum cell concentration, and cell specific consumption/production rates for main metabolites), the strategy was adapted to accommodate these differences. However, the feeding regime and rationale remained the same. This work is the

subject of chapter 5, presented in the form of an article submitted for publication to Journal of Biotechnology in 2024.

– Specific objective nº 3 –

Evaluate the developed process for the intensified production of a rVSV viral vector in high cell density cultures of HEK293 cells.

A generic feeding strategy should equally be applicable for the production of other viruses using the same cell substrate. As influenza (A/Puerto Rico/8/34 H1N1) was the model virus for this work, we have selected a viral vector, also produced in HEK293 cells, to validate this premise. A rVSV-vectored SARS-CoV-2 vaccine candidate (rVSVInd-msp-SF-Gtc) was chosen. This work is the subject of chapter 6, presented in the form of an article submitted for publication to Biotechnology Progress in 2024.

CHAPTER 4 ARTICLE 2 : INTENSIFIED INFLUENZA VIRUS PRODUCTION IN SUSPENSION HEK293SF CELL CULTURES OPERATED IN FED-BATCH OR PERFUSION WITH CONTINUOUS HARVEST

4.1 Article presentation

The article presented in this chapter was published on December 5, 2023 in Vaccines. Here, we report two intensified processes for influenza virus production using a well-established HEK293 cell line. The developed strategies, which focused on minimizing medium consumption, aimed to intensify virus production by increasing the cell density at infection and decreasing the retention time of viral particles in the culture broth.

To characterize the so-called “cell density effect”, typically linked to nutrient limitations and toxic by-product accumulation, the average cell specific consumption/production rates for main metabolites were calculated during different phases of reference cultures which displayed or not the effect. Based on this information, two different processes, characterized by low medium consumption, were designed to guarantee the supply of fresh nutrients and maintain cells in a desired metabolic state while sustaining high cell densities: a hybrid fed-batch with continuous harvest and a low PR perfusion. The developed processes were scaled up to a 3 L STR bioreactor and a TFDF system was employed as cell retention device. The capacity of the TFDF membrane for continuous virus harvesting was assessed for both the infection phase and the final harvest steps.

Additional information, including detailed schematics for the experimental set up, were included in section 4.11.

4.2 Intensified Influenza Virus Production in Suspension HEK293SF Cell Cultures Operated in Fed-Batch or Perfusion with Continuous Harvest

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4.3 Abstract

Major efforts in the intensification of cell culture-based viral vaccine manufacturing focus on the development of high-cell-density (HCD) processes, often operated in perfusion. While perfusion operations allow for higher viable cell densities and volumetric productivities, the high perfusion rates (PR) normally adopted—typically between 2 and 4 vessel volumes per day (VVD)—dramatically increase media consumption, resulting in a higher burden on the cell retention device and raising challenges for the handling and disposal of high volumes of media. In this study, we explore high inoculum fed-batch (HIFB) and low PR perfusion operations to intensify a cell culture-based process for influenza virus production while minimizing media consumption. To reduce product retention time in the bioreactor, produced viral particles were continuously harvested using a tangential flow depth filtration (TFDF) system as a cell retention device and harvest unit. The feeding strategies developed—a hybrid fed-batch with continuous harvest and a low PR perfusion—allowed for infections in the range of $8\text{--}10 \times 10^6$ cells/mL while maintaining cell-specific productivity comparable to the batch control, resulting in a global increase in the process productivity. Overall, our work demonstrates that feeding strategies that minimize media consumption are suitable for large-scale influenza vaccine production.

Keywords: influenza vaccine; process intensification; perfusion; fed-batch; suspension cells; high cell density; HEK293; continuous harvest

4.4 Introduction

Millions of people are afflicted with the flu every year, a disease resulting from an infection with an influenza virus that causes more than half a million deaths around the world, particularly amongst the more vulnerable groups of the population [1]. As vaccination is the most efficient protective measure against the disease, seasonal vaccination campaigns require millions of doses to be produced in short timelines, and billions of doses would be necessary to respond to a possible pandemic scenario [3, 6]. Our recent experience with the COVID-19 pandemic has shown that the development of different vaccine production platforms is essential to guarantee a worldwide supply of vaccines. In this scenario, cell culture-based processes emerge as a more flexible and responsive platform for influenza vaccine production and are a valuable alternative to traditional egg-based manufacturing systems [8]. Cell culture-based influenza vaccine manufacturing involves the production of whole viruses for inactivated vaccine formulations, which is achieved by cultivating

mammalian or avian cells to sufficiently high cell densities and infecting those cells with the desired virus strain. When compared to traditional egg-based systems, cell culture processes offer various advantages: (i) cell culture processes can be easily scaled up, particularly when employing cells grown in suspension, with operations in tightly controlled bioreactors of more than 2000 L [102]; (ii) less risk of introducing antigenic modifications due to adaptation of virus for growth in eggs [62, 63]; (iii) fast response to pandemic scenarios, as cell-based production can be accelerated by the use of high-cell-density (HCD) stocks and perfusion techniques [64, 103]. Additionally, the development of single-use systems has increased the flexibility of cell culture manufacturing plants while reducing the upfront capital costs for building a new facility [104].

HEK293 cells are widely accepted as an expression platform and have been extensively employed for the production of recombinant proteins and viral vectors [105-108]. A growing number of therapeutic agents produced using these cells are currently on the market and have been approved for use by the FDA and the EMA [105], including the COVID-19 vaccine developed by the University of Oxford and AstraZeneca (ChAdOx1 nCoV-19) [109], highlighting the safety of this cell substrate. HEK293 cells have been successfully adapted for growth in suspension and are capable of growing to high cell densities in animal component-free media [30, 106], characteristics that make them a valuable host for large-scale viral vaccine manufacturing.

In order to be consolidated as a cost-effective vaccine manufacturing system, cell culture-based processes need to be intensified, essentially by addressing two main factors: i) increasing the viable cell density (VCD) at the time of infection (TOI) while maintaining cells in a desired metabolic state, to increase final virus yield and avoid the so-called “cell density effect” (CDE)—characterized by a loss in cell-specific productivity (number of viruses produced per cell) when the infection occurs at higher cell densities [23, 66, 110]; ii) decreasing the residence time of the viral particles in the bioreactor to limit product degradation [25]. In order to sustain high cell concentrations and therefore increase process productivity, fed-batch (addition of a concentrated feed media) and perfusion strategies (continuous exchange of media with cells kept inside of the reactor by a cell retention device) are usually employed [21, 80, 111]. However, as nutrient and by-product concentrations have been shown to impact virus production, feeding strategies must be designed to maintain the cells in a desired metabolic state by keeping key nutrients/metabolites within defined thresholds to avoid limitations/inhibitions [15, 42, 112]. Metabolic flux analysis performed in HEK293 cells showed that the CDE observed for HCD cultures could be overcome

by keeping the metabolic profile of cells at similar levels to those of low-cell-density batch processes [110]. In that sense, the estimation of the average specific uptake/production rates for the main metabolites during the course of the process can be a simpler approach to evaluate the metabolic state of cells based on measurements of nutrient concentration and cell count and provide valuable information for the design and control of feeding strategies.

Successful influenza virus production in HCD processes operated in perfusion is reported [25-27, 29, 76]. While perfusion operations allow for higher viable cell densities and productivities [21], the high perfusion rates (PR) normally adopted—typically between two and four vessel volumes per day (VVD)—raises challenges for the preparation, handling, and disposal of high volumes of fluid [21]. Additionally, due to the higher flux through the membrane, high perfusion rates can increase the risk of progressive membrane fouling as well as increase the shear stress to which the cells are submitted. High inoculum fed-batch (HIFB) processes can drastically reduce the volume of media consumed while sustaining relatively high viable cell densities [102, 113, 114] and are typically applied when the production is limited by nutrient depletion instead of by-product accumulation. The combination of HIFB processes with a perfusion-based continuous harvest approach serves to reduce the retention time of viral particles in the reactor while alleviating by-product accumulation and offers the possibility of working with integrated continuous/semi-continuous processes [97]. Membrane-based cell retention devices, such as the alternating tangential flow (ATF) and tangential flow filtration (TFF) modules, have been extensively applied in perfusion processes for virus production [27-29, 38, 115, 116]. However, virus retention due to progressive membrane clogging has been reported for those modules for various membrane pore sizes [27, 37]. Tangential flow depth filtration (TFDF) membranes combine the advantages of both tangential filtration and depth filtration—the latter being typically applied as an alternative to centrifugation for the harvesting step in virus production processes—allowing for the processing of HCD cultures with minimal membrane fouling and high product recovery [32, 33, 117]. Additionally, TFDF modules are scalable and single-use and are currently commercially available for up to 2000 L bioreactor scale.

In this study, two scalable HCD processes, which explore HIFB and low perfusion rate operations, are developed for influenza A virus (IAV) production in suspension HEK293SF cells. The perfusion-based operations are performed using a TFDF system as a cell retention device and harvest unit. Feeding strategies were designed and evaluated based on the assessment of the

metabolic state of cells through the estimation of specific uptake/production rates for the main metabolites.

4.5 Materials and Methods

4.5.1 Cell Line and Culture Conditions

Suspension HEK293SF cells were kindly provided by the National Research Council Canada and were cultivated at 37 °C and 5% CO₂ in basal serum-free Xell HEK GM (Sartorius Stedim, Göttingen, Germany) medium supplemented with 6 mM L-Glutamine. Cells were maintained in 125 mL shake-tubes (25 mL working volume) using an orbital shaker at 135 rpm. Cell counting was performed using Vi-Cell XR Cell Viability analyser (Beckman Coulter, Pasadena, CA, USA). Analysis of main metabolite, glutamine (GLN), glutamate (GLU), glucose (GLUC), lactate (LAC), and ammonium (NH₄) was performed using a blood analyzer BioProfile® FLEX2 (Nova Biomedical, Waltham, MA, USA). Average specific uptake/production rates in a given interval for a metabolite S (q_s) were determined based on the following equations:

$$q_s = \frac{(V_n * S_n - V_{n-1} * S_{n-1}) - V_{perf} * (S_{perf} - S_{ave}) - V_{feed} * S_{feed}}{(t_n - t_{n-1}) * XV_{ave}} \quad (4.1)$$

$$S_{ave} = 0.5 * (S_{n-1} + S_{exp}) \quad (4.2)$$

$$XV_{ave} = 0.5 * (V_n X_n + V_{n-1} X_{n-1}) \quad (4.3)$$

where V_n is the culture volume at a given time (t_n), S_n is the concentration of a metabolite S at t_n , V_{perf} is the volume of perfused media in the given interval, S_{perf} is the concentration of metabolite S in the perfusion media, S_{ave} is the estimated average concentration of metabolite S in the reactor during the given interval, S_{exp} is the expected concentration of the metabolite S if the media was exchanged semi-continuously (for semi-continuous cultures, $S_{ave} = S_{n-1}$), V_{feed} is the volume of concentrated feed added in the given interval, S_{feed} is the concentration of metabolite S in the concentrated feeding media, XV_{ave} is the estimated average number of cells in the given interval, and X_n is the viable cell density at t_n .

Negative values of q_s indicate consumption of the nutrient/metabolite, while positive values indicate its production. However, to facilitate the discussions, comparisons between different consumption rates are made considering the absolute values.

The molar lactate-to-glucose ($Y_{LAC/GLUC}$) and ammonium-to-glutamine ratios ($Y_{NH4/GLN}$) were calculated as follows:

$$Y_{\frac{LAC}{GLUC}} = \frac{q_{LAC}}{-q_{GLUC}} \quad (4.4)$$

$$Y_{\frac{NH4}{GLN}} = \frac{q_{NH4}}{-q_{GLN}} \quad (4.5)$$

4.5.2 Virus Strains and Infection Conditions

Viral stocks of influenza A/Puerto-Rico/8/34 (H1N1), originally derived from ATCC's Global Bioresource Center, were adapted by serial passaging and produced in HEK293SF cells [77]. Infections were performed without media exchange, at a multiplicity of infection (MOI) of 10^{-3} with the addition of TPCK trypsin (Sigma, St. Louis, MO, USA) at a final concentration of 1 $\mu\text{g/mL}$. After infection, the temperature was shifted to 35 °C.

4.5.3 Feeding Strategies and Process Operation

Two different feeding strategies for HCD processes, aiming to minimize the volume of media consumed, were developed in small-scale experiments and scaled up to a 3 L benchtop bioreactor (see following sections for experimental setup). For the HIFB operation, cultures were inoculated at 2×10^6 cells/mL in basal media at 80% of the final working volume. Daily addition of concentrated, chemically defined and serum-free media CellBoost5 (Cytiva, Marlborough, MA, USA) supplemented with 30 mM L-glutamine was performed. The volume of concentrated feeding media to be added varied from 5 to 15% (v/v) of the culture volume and was determined based on the growth rate and the values for q_{GLN} (Equation (1)) for the previous sampling interval. Based on these values and using basic mass balance equations for viable cell density and nutrient concentration, a prediction for glutamine consumption for the next interval was made, and a corresponding volume of feed was added to replenish consumed glutamine and maintain its concentration above limiting values. After infection, performed at a cell density between 8 and 9×10^6 cells/mL, a continuous harvest of the produced virus was performed at a PR of 0.5 VVD with perfusion media (basal media supplemented with 10% v/v CellBoost5 and 8 mM L-glutamine). For the perfusion operations, cultures were inoculated at 1.5×10^6 cells/mL in basal media. A PR of 0.5 VVD was used for the small-scale experiments throughout the process using a perfusion media composed of basal media supplemented with 10% v/v CellBoost5 and 8 mM L-glutamine. On

bioreactor operations, the PR was adjusted based on cell metabolism to keep nutrients above minimum levels and to limit by-product accumulation, varying between 0.4 and 0.8 VVD. The perfusion rate and media supplementation were defined based on average values for growth rate and q_s (Equation (1)) obtained in preliminary experiments and basic mass balance equations for cell growth and nutrient consumption.

4.5.4 Small-Scale Experiments

Small-scale experiments were conducted in 50 mL shake-tubes (TPP Techno Plastic Products AG, Trasadingen, Switzerland) agitated at 180 rpm with an inclination of 45° in an orbital shaker incubator with an orbital throw of 25 mm. Cultures were maintained at an approximately constant working volume of 10 mL (some variations occurred due to culture sampling and addition of feed). Cells were infected between 2 and 10×10^6 cells/mL, depending on the tested strategy. In order to simulate both the perfusion and the continuous harvest operations, shake-tubes were operated in a pseudo-perfusion mode whereby, on a daily basis, tubes were centrifuged for 3 min at $300\times g$ and a previously determined volume of media, equivalent to 0.5 VVD, was removed and replenished with fresh perfusion media. All small-scale experiments were performed in duplicates.

4.5.5 Bioreactor Set-Up and Operation

A 3 L benchtop bioreactor (Applikon Biotechnology, Delft, The Netherlands) equipped with two marine impellers was used for all of the strategies tested. The bioreactor was maintained at 37 °C, pH 7.1, and stirring speed of 100 rpm. The temperature was shifted to 35 °C for the infection phase. Dissolved oxygen was controlled at 40% of air saturation via surface aeration and pure oxygen sparging through a micro-sparger. The bioreactor was equipped with a capacitance probe (Aber Instruments, Aberystwyth, UK) for biomass monitoring. A 30 cm² (pore size 2.0–5.0 µm) TFDF membrane (Repligen Corporation, Waltham, MA, USA) operated by the KML-100 system (Repligen Corporation, USA) was employed as cell retention device for perfusion and continuous harvest operations. The recirculation rate was maintained at 1 L/min, measured by an integrated flowmeter. Continuous addition of perfusion media was regulated by a weight control system to maintain bioreactor level according to the permeate flow rate. After infection, the permeate material was collected in ice-cooled bottles, which were exchanged at every sample point (permeate pool). At the time of harvest (TOH), determined based on the capacitance signal and on the culture viability, the bioreactors were harvested through the cell retention device. The final

harvest comprised a primary concentration step, followed by a diafiltration and a final concentration, which resulted in a 20% increase in the final volume. To avoid membrane clogging, the recirculation rate was increased to 2 L/min prior to harvesting.

4.5.6 Virus Quantification and Productivity Evaluation

Hemagglutinin (HA) content was evaluated using hemagglutination assay based on the virus's capacity to reticulate red blood cells. Briefly, serial-diluted samples were incubated with purified chicken erythrocytes (Innovative Research, Novi, MI, USA) at room temperature for 2–12 h. Plates were read based on the presence of a pellet of sedimented red blood cells. Quantification of infectious virus particles (IVP) was performed using TCID₅₀. Briefly, adherent MDCK cells were cultured in VP-SFM culture medium (Gibco, New York, NY, USA) supplemented with 1% Pen/Strep (Gibco, USA) in a 96-well plate at a seeding density of 0.2×10^6 cells/well. After 24 h, the culture media was exchanged for trypsin-containing medium (1 µg/mL) and infected with 5-fold serial dilutions of infectious culture supernatant. Plates were incubated for 4–7 days at 37 °C and evaluated for the presence/absence of cytopathic effect. TCID titers were calculated following the Reed–Muench Method [118].

Three productivity parameters were selected to compare the different production processes and were calculated based on the total virus yield at a given time (Y_T) using the hemagglutination titers:

$$Y_T = \left[C_R + \sum_0^t (C_P * PR) \right] * V_R \quad (4.6)$$

where C_R is the concentration of virus in the reactor at a given time, C_P is the concentration of virus in the permeate pool, PR is the perfusion rate, and V_R is the reactor working volume.

The cell-specific virus yield (CSVY), the space–time yield (STY), and the yield on media (Y/M) were calculated as follows:

$$CSVY = \frac{Y_T}{X_T * V_R} \quad (4.7)$$

$$STY = \frac{Y_T}{V_R * t_T} \quad (4.8)$$

$$\frac{Y}{M} = \frac{Y_T}{V_T} \quad (4.9)$$

where X_T is the maximum total cell concentration, t_T is the total culture duration, and V_T is the total volume of media consumed.

4.6 Results

4.6.1 Small-Scale Characterization of the CDE

In order to characterize the effect of potential nutrient limitations in HCD processes for IAV production in HEK293 cells, three operation strategies were tested in small-scale experiments: a low-cell-density batch control (ST-B) infected at 2×10^6 cells/mL, a medium-cell-density batch (ST-B_M) infected at 4×10^6 cells/mL, and a high-cell-density pseudo-perfusion (ST-P_H) with a fixed PR of 0.5 VVD (non-supplemented basal media) infected at 13×10^6 cells/mL. All of the experimental conditions tested are summarized in Table 4.1. During the 24 h that followed the infection, cells continued growing for all three conditions tested, reaching 3.2×10^6 cells/mL for the ST-B culture, 8.1×10^6 cells/mL for ST-B_M, and 20.5×10^6 cells/mL for ST-P_H (Figure 4.1A). As expected, a clear drop in CSVY was observed for both ST-B_M and ST-P_H strategies when compared to the low-cell-density batch cultures (Figure 4.1B). The drop in CSVY was reflected in a lower Y/M and a decrease, or no significant increase, in the STY . Average cell-specific uptake/production rates for the main metabolites (q_s) were assessed before and after infection based on mass balance equations. The average specific uptake rate of glutamine (q_{GLN}), as well as the specific production of ammonium (q_{NH4}), was significantly lower both before and after infection for the medium and high-cell-density strategies (Figure 4.1A). This decrease in the uptake rate is most likely linked to the low residual concentrations of this nutrient in the culture media [119], which was below 1 mM at the TOI for both cultures (Figure A1). Interestingly, although residual glutamine concentrations were lower at 24 hpi (hours post-infection) for both ST-B_M and ST-P_H cultures—below 0.5 mM compared to 2.4 mM for the ST-B cultures (Figure A1)—their respective average $Y_{NH4/GLN}$ for the 24 h following the infection was higher (Table 2), indicating less efficient glutamine metabolism after infection. While the average specific uptake of glucose (q_{GLUC}) was also lower both before and after infection for the medium- and high-cell-density cultures, the difference between those and that of the ST-B batch control was less pronounced, even though the residual concentrations of this nutrient were significantly different—6.5 mM and 9.5 mM, respectively, for the ST-B_M and ST-P_H cultures, and 30 mM for ST-B. For this reason, the feeding strategies for the HCD processes were mainly designed based on specific glutamine consumption.

Table 4.1 Summary of culture conditions and results.

Run Code	ST-B	ST-B _M	ST-P _H	ST-FB _{HCD}	ST-P _{HCD}	BR-B	BR-FB _{HCD}	BR-P _{HCD}
V _R (mL)	10	10	10	10	10	1800	2000	2000
Growth phase	Batch	Batch	Perfusion ^c (0.5 VVD)	Fed-batch	Perfusion ^c (0.5 VVD)	Batch	Fed-batch	Perfusion (0.4–0.8 VVD)
Infection phase	Batch	Batch	Perfusion ^c (0.5 VVD)	Perfusion ^c (0.5 VVD)	Perfusion ^c (0.5 VVD)	Batch	Perfusion (0.5 VVD)	Perfusion (0.5 VVD)
VCD ₀ (10 ⁶ cells/mL)	0.35	0.35	0.35	2	1.5	0.5	2	1.5
VCD _{TOI} (10 ⁶ cells/mL)	2	4	13	8–10	8–10	1.6	8.5	8.6
VCD _{MAX} (10 ⁶ cells/mL) ^a	3.2 ± 0.16	8.1 ± 0.4	20.5 ± 2.16	14.3 ± 0.08	15.0 ± 0.05	2.4 ± 0.16	10.1 ± 0.42	10 ± 0.5
HA _{TOH} (logHAU/mL)	3.5	3.1	3.65	4.11	4.26	3.7	4.2	4
TCID _{TOH} (10 ⁸ IVP/mL) ^a	0.86 ± 0.15	-	-	5.28 ± 0.07	6.75 ± 1.5	0.53 ± 0.1	1.01	1.14 ± 0.21
HA _{Tot} (HAU) ^b	(3.20 ± 0.2) × 10 ⁴	(1.28 ± 0.3) × 10 ⁴	(5.44 ± 0.1) × 10 ⁴	(1.30 ± 0.01) × 10 ⁵	(1.86 ± 0.01) × 10 ⁵	8.1 × 10 ⁶	3.4 × 10 ⁷	2.4 × 10 ⁷
Y/M (10 ⁶ HAU/L) ^b	3.20 ± 0.06	1.28 ± 0.2	1.53 ± 0.08	6.51 ± 0.04	6.45 ± 0.06	4.53	7.81	2.98
CSVY (HAU/10 ⁶ cell) ^b	917 ± 12	122 ± 17	209 ± 35	834 ± 2	1129 ± 7	1311	1268	1220
CSVY _{TCID} (IVP/cell) ^b	24.7 ± 4	-	-	-	-	15	-	14
STY (10 ⁶ HAU/L/day) ^b	0.64 ± 0.01	0.18 ± 0.01	0.68 ± 0.01	2.60 ± 0.03	3.72 ± 0.02	1.1	3	2.3
Total media	1 × V _R	1 × V _R	4 × V _R	2 × V _R	2.9 × V _R	1 × V _R	2.2 × V _R	4 × V _R

Abbreviations: VCD₀, initial viable cell density; VCD_{TOI}, viable cell density at time of infection; VCD_{MAX}, maximum viable cell density; HA_{TOH}, HA content at time of harvest; TCID_{TOH}, infectious virus titer at time of harvest; HA_{Tot}, total yield based on HA content; Y/M, yield on media based on HA content; CSVY, cell-specific virus yield based on HA content; CSVY_{TCID}, cell-specific infectious virus yield; STY, space–time yield based on HA content; V_R, reactor working volume.

^aStandard deviation was calculated based on the biological duplicates for ST cultures and based on duplicates for the same time point for BR cultures.

^bStandard deviation for productivity parameters was calculated based on biological duplicates for ST cultures.

^cPerfusion operations were performed semi-continuously (pseudo-perfusion) for all shake-tube experiments.

Table 4.2 Average values of $Y_{\text{LAC/GLUC}}$ and $Y_{\text{NH}_4/\text{GLN}}$ in the 48 h prior to the infection (−48 hpi) and 24 h following the infection (24 hpi) for all cultures.

		ST-B	ST-B _M	ST-P _H	ST-FB _{HCD}	ST-P _{HCD}	BR-B	BR-FB _{HCD}	BR-P _{HCD}
$Y_{\text{LAC/GLUC}}$	−48 hpi	1.75 ± 0.4	0.2 ± 0.04	0.57 ± 0.002	0.06 ± 0.00	0.73 ± 0.02	1.58	0.78	1.29
	24 hpi	0.31 ± 0.04	0.87 ± 0.12	0.59 ± 0.15	0.32 ± 0.05	0.31 ± 0.01	1.64	1.24	1.46
$Y_{\text{NH}_4/\text{GLN}}$	−48 hpi	0.51 ± 0.05	0.07 ± 0.00	0.29 ± 0.1	0.22 ± 0.01	0.31 ± 0.001	0.53	0.01	0.2
	24 hpi	0.09 ± 0.03	2.97 ± 0.44	0.83 ± 0.01	0.06 ± 0.01	0.16 ± 0.001	0.06	0.47	0.12

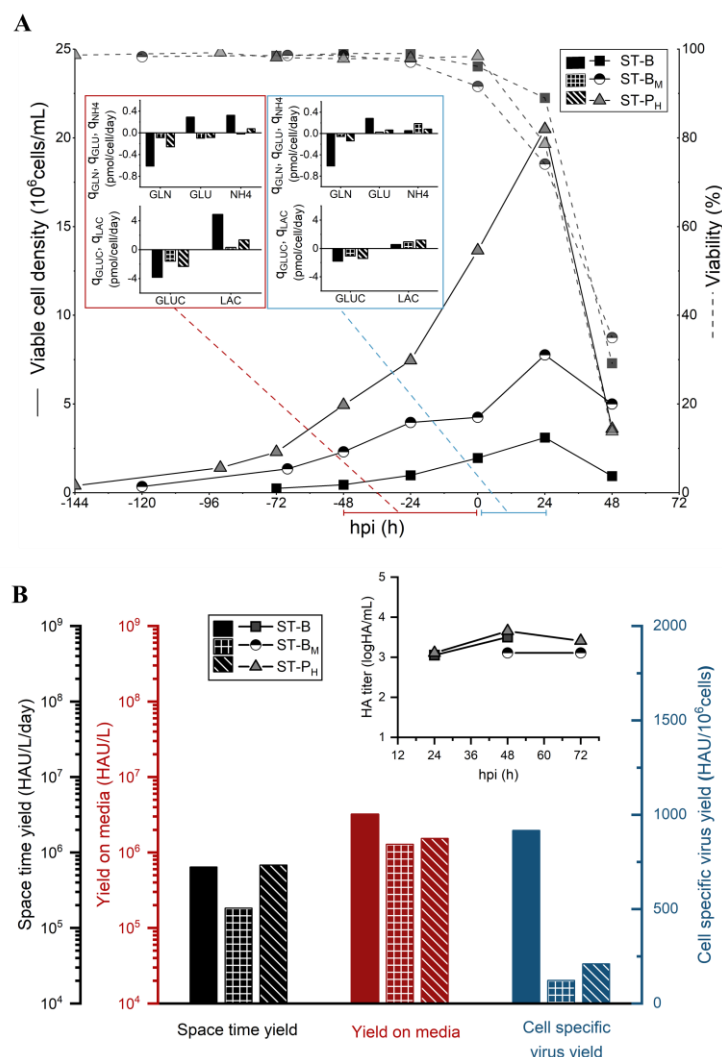


Figure 4.1 **(A)** Comparison of cell growth, viability, and average specific consumption/production rates (q_s) of main metabolites and **(B)** productivity of 50 mL shake-tube cultures of HEK293SF cells for influenza virus production, consisting of low-cell-density batch (ST-B, infected at 2×10^6 cells/mL), medium-cell-density batch (ST-B_M, infected at 4×10^6 cells/mL), and high-cell-density pseudo-perfusion, 0.5 VVD, (ST-P_H, infected at 13×10^6 cells/mL). Insets show the average cell-specific rates before and after infection.

4.6.2 Small-Scale Development of HCD Feeding Strategies

Based on the average consumption/production rates observed for the low-cell-density batch process, two HCD feeding strategies, targeting to minimize the volume of media consumed while keeping the uptake/production profiles similar to the batch control, were designed and evaluated in shake-tubes: a hybrid fed-batch with continuous harvest (ST-FB_{HCD}) and a perfusion strategy (ST-P_{HCD}), both described in detail in Table 4.1. In order to supply other nutrients and metabolites that

were not quantified, a concentrated feeding media, Cell Boost 5, was chosen for the bolus feeding additions in the fed-batch strategy and to supplement the perfusion media for the perfusion-based strategy. The amount of concentrated feed to be added was determined based on glutamine consumption, following the assumption that those non-quantified metabolites would be consumed at a similar rate. ST-FB_{HCD} and ST-P_{HCD} cultures reached, on average, 14.3×10^6 cells/mL and 15×10^6 cells/mL, respectively, at 24 hpi (Figure 4.2). Unlike what was observed for the non-optimized cultures (ST-B_M and ST-P_H), the average q_{GLN} was comparable between the HCD cultures and the low-cell-density batch control (ST-B), both before and after infection (Figure 4.2). While $Y_{NH4/GLN}$ was higher before infection for the ST-B culture, indicating a less efficient glutamine metabolism for the cultures operated in batch, cultures in all three conditions reached similar levels for this ratio in the 24 h that followed the infection (Table 2). Although average q_{GLUC} was less important before infection for both HCD cultures, q_{LAC} was also smaller, which was reflected in lower values of $Y_{LAC/GLUC}$ for those cultures (Table 2), indicating a more efficient use of the sugar. It is worth noting that glucose concentrations at the TOI were above 15 mM for both ST-P_{HCD} and ST-FB_{HCD} (Figure A2), showing that no limitations of this nutrient occurred. After infection, q_{GLUC} and q_{LAC} attained similar levels for both the HCD cultures (ST-P_{HCD} and ST-FB_{HCD}) and the ST-B control.

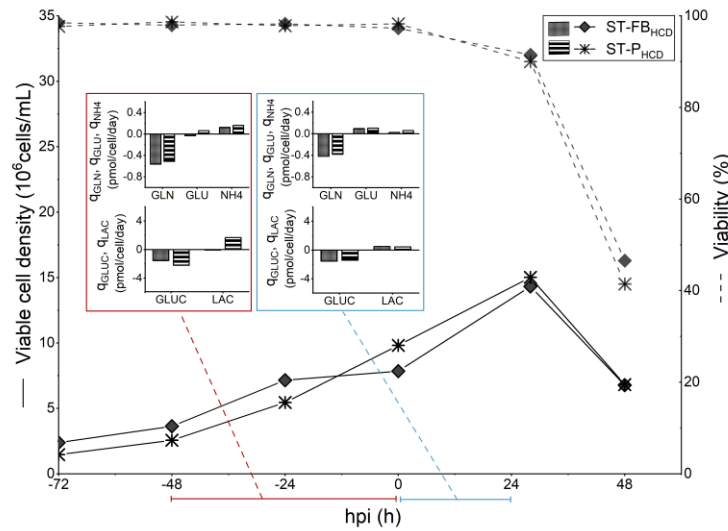


Figure 4.2 Comparison of cell growth, viability, and average specific consumption/production rates (q_s) of main metabolites for 50 mL shake-tube cultures of HEK293SF cells for influenza virus production, consisting of HCD hybrid fed-batch/pseudo-perfusion (ST-FB_{HCD}, infected at 8.5×10^6 cells/mL) and HCD pseudo-perfusion (ST-P_{HCD}, infected at 10×10^6 cells/mL).

With respect to virus production, HA content at TOH (48 hpi) was, respectively, 0.6- and 0.8-log higher for the ST-FB_{HCD} and ST-P_{HCD} cultures (Table 4.1). The CSVY was comparable for all three conditions (Figure 4.3), demonstrating that the CDE was successfully overcome. Even though the developed HCD feeding strategies resulted in a higher volume of media consumed, the *Y/M* was 2-fold higher, and the *STY* was, respectively, 4- and 6-fold higher for the HCD fed-batch and perfusion strategies, in comparison to the batch control. In order to better characterize the produced material, the infectious titer at TOH was determined for the different conditions tested (Table 4.1). The infectious titer at TOH was, respectively, 6- and 7-fold higher for the ST-FB_{HCD} and ST-P_{HCD} cultures when compared to the batch control, which suggests that the material produced by the HCD cultures kept its infectivity.

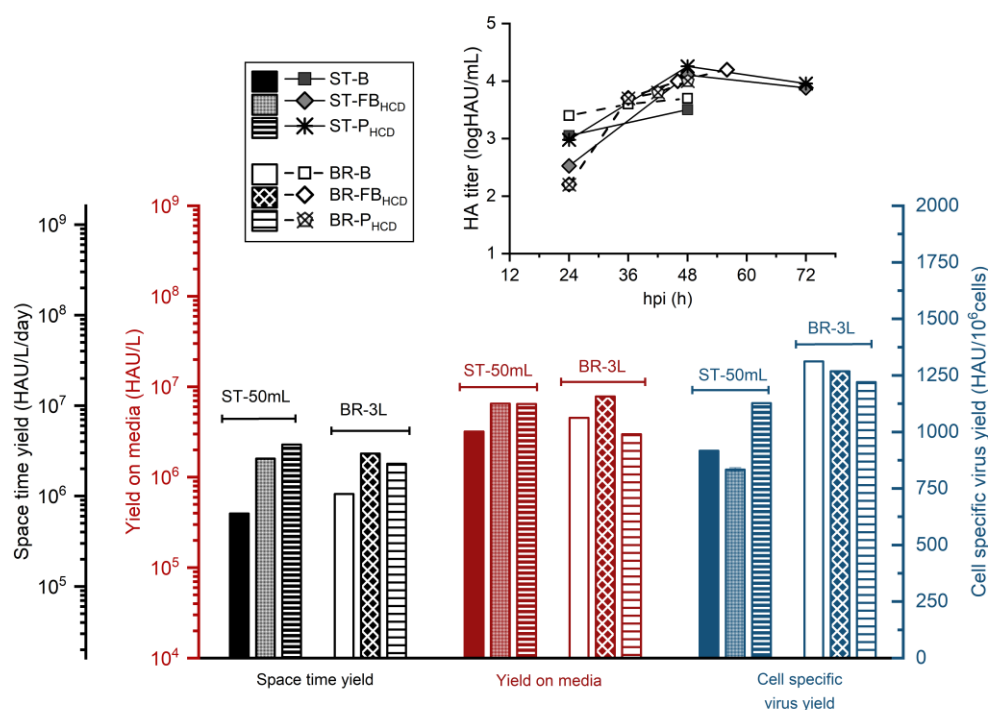


Figure 4.3 Virus titers and productivity of 50 mL shake-tube (ST) and 3 L bioreactor (BR) cultures of HEK293SF cells for influenza virus production, operated in low-cell-density batch (B, infected at 2×10^6 cells/mL), HCD hybrid fed-batch/perfusion (FB_{HCD}, infected at 8.5×10^6 cells/mL), and HCD perfusion (P_{HCD}, infected between 8.5 – 10×10^6 cells/mL). Perfusion operations in shake-tube cultures were performed semi-continuously (pseudo-perfusion).

4.6.3 Scale Up to 3 L STR Bioreactor

To evaluate the scalability of the designed strategies, two perfusion-based runs were performed in a 3 L STR bioreactor, using a TFDF system as the cell retention device. A scale-up of the batch run was also performed for comparison, and the TFDF system was employed for the harvest step. The feeding profile for both HCD runs is presented in Figure 4.4. For the hybrid fed-batch strategy (BR-FB_{HCD}), the bioreactor was inoculated at 2×10^6 cells/mL. The addition of concentrated feeding media was performed approximately every 16 h during the growth phase (Figure 4.4), and the volume added was determined based on specific consumption/production rates for glutamine, as defined in the small-scale experiments. After infection, the culture was shifted to perfusion mode with a fixed PR of 0.5 VVD to continuously harvest the produced virus. Since the perfusion was kept off during the 4 h that followed the infection—so as to ensure virus entry into the cells—the equivalent volume of media for that time period was exchanged right before the infection. Cells were infected at 8.5×10^6 cells/mL and, differently from what was observed for the same strategy performed in shake-tubes, the cell-specific growth rate decreased drastically after infection, with cells reaching a maximum concentration of 10.1×10^6 cells/mL at 35 hpi (Figure 4.5A). Nevertheless, the viability profile and the growth profile before infection were comparable for both scales. Metabolic profiles greatly varied for the two different production scales. While glutamine consumption was similar during the growth phase, bioreactor cultures presented higher average q_{GLUC} and q_{LAC} before and after the infection. Additionally, the glucose metabolism was less efficient for the BR-FB_{HCD} culture in comparison to the ST-FB_{HCD} cultures, with values of $Y_{LAC/GLUC}$ 13-fold and 4-fold higher for the first, respectively, before and after infection. That led to lactate accumulation up to 55 mM right before infection for the bioreactor culture, compared to 10 mM for the equivalent shake-tube cultures (Figure A3). This discrepancy in the metabolism between both production scales could be related to bioreactor operation parameters, particularly DO and pH control [120]. Despite the differences in growth and metabolism, HA content from the bioreactor was similar to the small-scale model throughout the run (Figure 4.3), and although it was slightly lower for the BR-FB_{HCD}, the overall difference was within the assay error (0.15 logHAU/mL). Consequently, values for STY and Y/M were slightly lower for the bioreactor run but remained comparable. On the other hand, the $CSVY$ was significantly higher for the bioreactor culture—1268 HAU/ 10^6 cells for the BR-FB_{HCD} compared to 834 HAU/ 10^6 cells for ST-FB_{HCD}—as the HA content was comparable throughout the run between both scales even though the

maximum cell density reached was lower for the bioreactor. However, the infectious titer at TOH was lower for the BR-FB_{HCD} (Table 4.1). While this decrease in infectivity could be related to the accumulation of lactate in the bioreactor, it could also be partially related to the difference between the semi-continuous operation in the shake-tubes and the continuous harvest from the bioreactor.

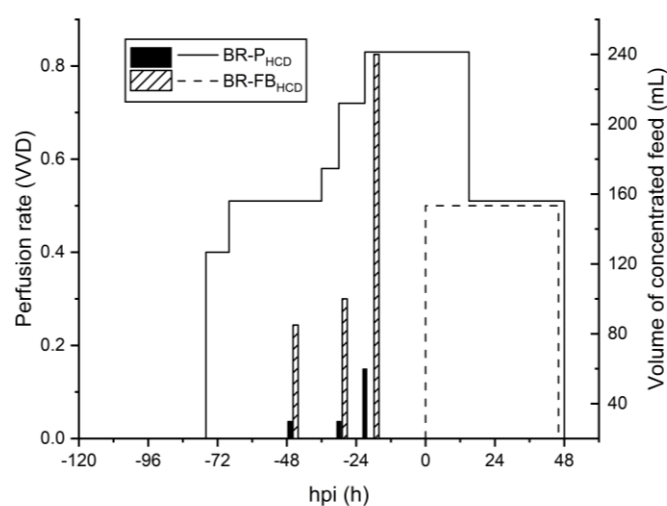


Figure 4.4 Perfusion rate (lines) and volume of concentrated feed (bars) for 3 L bioreactor cultures operated in perfusion (BR-P_{HCD}) and hybrid fed-batch/perfusion (BR-FB_{HCD}) for influenza virus production in suspension HEK293SF cells.

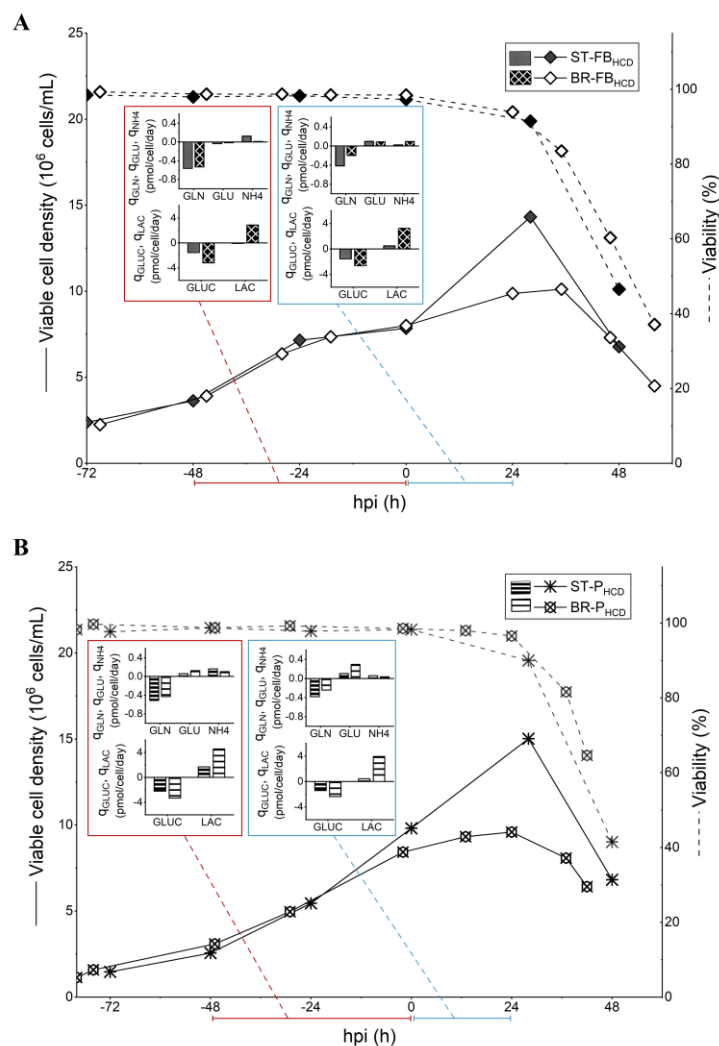


Figure 4.5 Comparison of cell growth, viability, and average specific consumption/production rates (q_s) of main metabolites for 50 mL shake-tube (ST) and 3 L STR bioreactor (BR) cultures of HEK293SF cells producing influenza virus, operated in (A) fed-batch (FB_{HCD}) and (B) perfusion (P_{HCD}). Perfusion operations in shake-tube cultures were performed semi-continuously (pseudo-perfusion).

For the intensified perfusion strategy (BR-P_{HCD}), the bioreactor was inoculated at 1.5×10^6 cells/mL. Since previous attempts to scale up this strategy showed that differences in the metabolism and nutrient supply, mainly regarding the glucose metabolism, between the semi-continuous small-scale model and the continuous perfusion bioreactor resulted in nutrient limitations and lower titers for the latter, a different strategy was applied for the feeding and the PR control. A stepwise increase of 20% in the PR was performed based on nutrient concentration and specific consumption/production rates for the main metabolites. During the course of the run,

the PR varied from 0.4 to 0.8 VVD (Figure 4.4), with an average PR of 0.56 VVD. Concentrated feeding was added to the bioreactor as a separated stream—instead of directly supplemented in the perfusion media—and the volume to be added was defined based on specific glutamine and glucose consumption. The bioreactor was infected at 8.6×10^6 cells/mL and, similarly to what was observed for the hybrid fed-batch strategy, cell growth drastically decreased after infection, with cells reaching a maximum cell concentration of 10×10^6 cells/mL—compared to 15×10^6 cells/mL for the same strategy performed in shake-tubes (Figure 4.5B). The BR- P_{HCD} culture presented, respectively, higher specific uptake and production rates for glucose and lactate both before and after the infection when compared to the ST- P_{HCD} cultures (Figure 4.5B). That led to a lactate concentration of 38 mM right before infection, compared to 15 mM for the semi-continuous shake-tube cultures (Figure A3). Regarding virus production, HA content was slightly lower for the bioreactor run in comparison to the same strategy performed in shake-tubes (Figure 4.3), which resulted in an overall lower productivity for the BR- P_{HCD} culture (Table 4.1). The different feeding regimes for the PR resulted in a higher consumption of media for the bioreactor culture— $4 \times V_R$ for the BR- P_{HCD} compared to $2.9 \times V_R$ for the ST- P_{HCD} cultures—which resulted in a 2-fold lower Y/M for the first. As observed for the hybrid fed-batch strategy, the $CSVY$ was higher for the bioreactor culture, at 1220 HAU/ 10^6 cells for the BR- P_{HCD} and 1129 HAU/ 10^6 cells for the ST- P_{HCD} , which can also be related to the lower maximum cell density reached in the bioreactor.

For scaling up the batch operation, the bioreactor was inoculated at 0.25×10^6 cells/mL, and the infection was performed at 1.6×10^6 cells/mL around 3 days later. Interestingly, the same reduction in cell growth after infection was observed, with a maximum VCD of 2.4×10^6 cells/mL compared to 3.5×10^6 cells/mL for the ST-B run (Figure 4.6). As the recirculation of the culture through the TFDF membrane was not started until the harvest, this decrease in the growth rate is most probably not related to shear stress in the membrane module but rather to the bioreactor operation and control. As was observed for the previously discussed bioreactor runs, specific glucose uptake and lactate production were higher for the bioreactor (BR-B) throughout the run when compared to the shake-tube cultures operated in batch (ST-B). Furthermore, even though the glucose concentration was 2-fold lower at infection for the BR-B (Figure A3), average specific lactate production in the 24 h that followed the infection was 15-fold higher for the BR-B culture and glucose uptake was 3-fold higher for the same base of comparison (Figure 4.6). These results indicate that the metabolism of cells in the bioreactor was much less efficient than that of cells cultured in shake-

tubes, and as previously discussed, these differences are most likely linked to oxygen supply and/or pH control. Nonetheless, final HA content was higher for the bioreactor run at 3.7 logHAU/mL at TOH compared to 3.5 logHAU/mL at TOH for the ST-B culture (Table 4.1), which resulted in an overall higher productivity for the bioreactor (Figure 4.3). However, infectious virus titer at TOH was almost 2-fold lower for the BR-B run, and this decrease in infectivity could be related to the higher lactate concentration attained in the bioreactor, 50 mM in comparison to 19 mM for the shake-tube batch cultures (Figure A3).

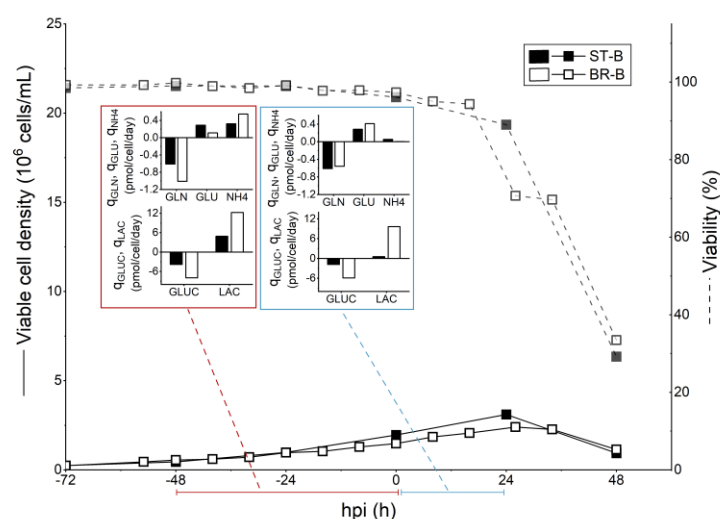


Figure 4.6 Comparison of cell growth, viability, and average specific consumption/production rates (q_s) of main metabolites for 50 mL shake-tube (ST) and 3 L bioreactor (BR) cultures of HEK293SF cells producing influenza virus, operated in batch.

Regarding the productivity of all three bioreactor runs, $CSVY$ values were comparable between the HCD strategies and the low-cell-density batch—at 1311 HAU/10⁶ cells for BR-B, 1268 HAU/10⁶ cells for BR-FB_{HCD}, and 1220 for BR-P_{HCD} (Table 4.1). $CSVY$ values calculated based on the infectious virus titers ($CSVY_{TCID}$) for the batch and perfusion bioreactors were also comparable, at 15 IVP/cell for BR-B and 14 IVP/cell for BR-P_{HCD} (Table 4.1). Because of the higher consumption of media during the perfusion run, the Y/M was 1.5-fold lower for the BR-P_{HCD} culture in comparison to the batch control. On the other hand, the Y/M was 1.7-fold higher for BR-FB_{HCD} when compared to the batch control. Values of STY were, respectively, 2.7 and 2.1-fold higher for BR-FB_{HCD} and BR-P_{HCD} in relation to the BR-B run. Overall, these results show that the CDE was successfully overcome by the designed feeding strategies on the bioreactor scale, and the increase

in productivity for the developed HCD processes was maintained in the scale-up. However, the bioreactor operating parameters could potentially be further optimized so as to maintain cells in a more efficient metabolic state and maximize production.

4.6.4 IAV Production and Harvest Using the TFDF System

In this study, a TFDF membrane module was used as the cell retention device for the perfusion runs, as well as the harvest unit for all bioreactor runs. To evaluate virus retention during the course of the perfusion-based runs, samples from the bioreactor content and permeate were routinely taken and quantified. HA content for different time points after infection is presented in Figure 4.7. For both perfusion-based bioreactor runs, BR-P_{HCD} and BR-FB_{HCD}, HA content from the bioreactor and permeate samples were the same or remained within the assay error (0.15 logHAU/mL) throughout the infection phase, indicating that little to no virus was being retained in the bioreactor or being lost inside the membrane module. As to the perfusion bioreactor, BR-P_{HCD}, samples taken at 48 hpi were quantified by TCID₅₀, and infectious titers for the bioreactor broth and permeate were comparable, respectively, at 1.14×10^8 IVP/mL and 1.12×10^8 IVP/mL. A final harvest step was carried out for all three bioreactor runs, using the same membrane module that was employed during the perfusion phase for the BR-P_{HCD} and BR-FB_{HCD} runs, while using a new membrane for the BR-B batch run. As the harvest process through the TFDF system comprises a short diafiltration step, an increase in the harvest volume of 15–20% is expected. The total virus yield in the bioreactor (previous to the harvest step) and the total yield in the harvested volume are presented in Table 4.3. Total infectious yield and total HA content indicate that no virus retention nor virus loss occurred during the harvesting step for all three bioreactor runs.

Table 4.3 Volume and virus content before and after harvest through a TFDF membrane module.

	Volume (mL)		Virus Yield (logHAU)		Virus Yield (IVP)	
	Bioreactor (TOH)	TFDF Harvest	Bioreactor (TOH)	TFDF Harvest	Bioreactor (TOH)	TFDF Harvest
BR-B	1720	1950	6.89	6.95	7.9×10^{10}	5.66×10^{10}
BR-FB _{HCD}	2100	2510	7.48	7.49	2.1×10^{11}	2.3×10^{11}
BR-P _{HCD}	1900	2150	7.29	7.18	2.2×10^{11}	1.7×10^{11}

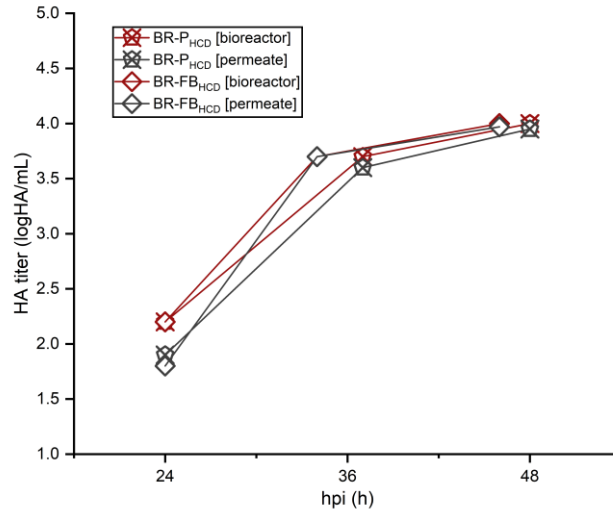


Figure 4.7 HA content in the bioreactor broth (red) and in the permeate (gray) for 3 L bioreactor cultures of HEK293SF cells operated in fed-batch (BR-FB_{HCD}) and perfusion (BR-P_{HCD}).

4.7 Discussion

4.7.1 Small-Scale Characterization of the CDE

Previous studies have shown that the CDE observed in HCD virus production processes could be successfully overcome with adequate feeding strategies, and that it could be related to nutrient limitation and inhibition by toxic by-products [23, 27, 29]. Metabolic flux analysis of suspension HEK293 cell cultures producing adenovirus in low-cell-density and HCD processes operated in batch and perfusion indicated that cell-specific virus productivity was maintained when feeding strategies were able to keep metabolic flux distribution profiles of HCD cultures at similar levels to low-cell-density processes [110]. In the present study, the metabolic state of cells in different stages of the process was estimated based on the average specific uptake/production rates for main metabolites, which were calculated through basic mass balance equations (Equation (1)). The sharp decrease in *CSVY* (Figure 4.1B) observed for ST-B_M and ST-P_H conditions was associated with reduced values of q_{GLN} and q_{NH4} before and after infection, which are most probably related to the low residual concentration of glutamine around the TOI for those cultures. Regarding glucose metabolism, although the residual concentration of the sugar was much lower for those cultures around the TOI, the difference between the values of q_{GLUC} and q_{LAC} was less pronounced for those in relation to the batch control, notably after infection (Figure 4.1A). These results suggest that

glutamine metabolism, both before and after infection, plays an important role in the CDE observed in HEK293SF cell cultures infected at higher cell densities.

4.7.2 Small-Scale Development of HCD Feeding Strategies

In this study, two feeding strategies were developed in small-scale experiments: a hybrid fed-batch with continuous harvest (ST-FB_{HCD}) and a low PR perfusion (ST-P_{HCD}). Feeding strategies were designed based on average metabolite rates to replenish consumed nutrients without overfeeding. Both feeding strategies were able to maintain the *CSVY* of cultures infected at 9×10^6 cells/mL at comparable levels to that of batch cultures infected at 2×10^6 cells/mL (Figure 4.3). While average q_{GLN} and q_{NH4} were at similar levels between the HCD cultures and the batch control both before and after infection, q_{GLUC} was around 2-fold lower for HCD cultures in the 48 h prior to infection (Figure 4.2). These results support the conclusion that, although glucose supply remains critical, glutamine metabolism has an important part in IAV production in HEK293SF cells.

The molar lactate to glucose ratio ($Y_{LAC/GLUC}$) was lower for both HCD strategies and, while this might indicate a more efficient use of glucose—which was also reported by Henry et al. [110] in HCD cultures producing adenoviral vectors—it may also be related to the accumulation of lactate in the culture media, which has been shown to play a role in shifting the metabolism towards lactate consumption [121]. In fact, the average q_{LAC} was negative on the 48 h prior to infection for ST-FB_{HCD} cultures, at around -0.08 pmol/cell/day, indicating that lactate was being consumed by the cells (Figure 4.2).

4.7.3 Scale Up to 3 L STR Bioreactor

The scale-up of the developed HCD feeding strategies was performed in a 3 L STR bioreactor employing a TFDF system as a cell retention device. While growth and viability profiles were similar between the two production scales during the growth phase for all three operation modes (batch, hybrid fed-batch, and perfusion), all three bioreactor cultures showed a sharp decrease in growth rate after infection (Figure 4.5 and Figure 4.6). As infected cells are reportedly more susceptible to shear stress [122], this reduction in cell growth after infection could be related to higher shear stress for the bioreactor cultures. On the other hand, differences in the operation and control, such as oxygen transfer and pH control, could also result in variations in metabolism and growth [120]. In fact, while glutamine consumption (q_{GLN}) and its molar ratio to ammonium

($Y_{\text{NH}_4/\text{GLN}}$) were overall comparable for the same feeding strategies in the different production scales (50 mL shake-tubes and 3 L STR bioreactor), glucose uptake and lactate production were much higher for bioreactor cultures. Furthermore, glucose metabolism was less efficient both before and after infection for bioreactor cultures, with $Y_{\text{LAC}/\text{GLUC}}$ values around 5-fold higher (Table 2), which led to increased lactate accumulation. These differences in metabolism could be related to the bioreactor operation parameters, notably the oxygen supply. A study performed by Zupke et al. [120] on hybridoma cells has shown that the flux of pyruvate to the TCA cycle greatly decreases in cells growing at low dissolved oxygen concentrations, resulting in higher glucose uptake and lactate accumulation. Nonetheless, virus titers and productivity remained comparable between the different production scales for all three operation modes (Figure 4.3). Both developed HCD feeding strategies were able to overcome the CDE when applied at the 3 L bioreactor scale, which resulted in an overall increase in the process productivity, notably for the hybrid fed-batch operation (1.7-fold higher Y/M and 2.7-fold higher STY when compared to the batch run). Other studies on the intensification of IAV production in different cell lines also reported successfully overcoming the CDE in HCD cultures through the development of perfusion-based feeding strategies employing higher perfusion rates [26, 27, 29, 76].

With respect to the virus titers obtained, the HA contents attained with the two HCD processes developed are comparable to what was reported with other suspension cell lines [27, 29, 76] but lower than what has been reported for suspension MDCK cells [123], known to be one of the preferable hosts for influenza production. While the infectious titers achieved in the present study are lower than what was previously reported for other HCD processes for IAV production [25-27, 76], this discrepancy is most likely related to the assay conditions, such as temperature shift, cell line, and use of crystal violet stain or specific antibodies. In fact, a study performed using the same cell line for IAV production reported much higher infectious titers for samples with similar or lower HA content [25]. Therefore, direct comparisons of virus titers between different studies were avoided.

While the concentration of host cell DNA and proteins—the main contaminants in cell culture-based vaccine preparations—was not assessed for the different processes developed in the present study, it is expected that higher cell densities at infection result in higher concentrations of those contaminants in the produced material. Therefore, the development of robust downstream processes is critical to support cell culture-based influenza vaccines. A robust and scalable process

for the purification of influenza viruses produced in HEK293SF cells is described [124], reporting up to 96.5% and 99.7% removal of host cell DNA and proteins, respectively.

4.7.4 IAV Production and Harvest Using the TFDF System

No virus retention was observed on all three bioreactor runs employing the TFDF system, neither during the production phase nor during the harvesting step (Figure 4.7 and Table 4.3). Other membrane-based cell retention devices, notably the ATF system, have also been employed for IAV production, but virus retention due to progressive membrane clogging was reported for membrane pores sizes $< 0.5\mu\text{m}$ [27, 29]. As for all membrane-based cell retention devices, shear stress might be an issue with the TFDF system, but optimization of culture parameters such as the recirculation rate can alleviate its effects. In this study, no detrimental effect of shear stress over virus production was observed, neither during the infection phase nor during the final harvest step. Other cell retention devices, notably acoustic settlers and inclined settlers, have also been applied for virus production [25, 26, 76], with the advantage of no virus retention and low shear stress, the latter being critical during the infection phase as infected cells tend to be more shear-sensitive [26, 122]. However, poor scalability due to heat exchange limitations is the main drawback of this kind of device. The TFDF system, on the other hand, is scalable and could support large-scale influenza vaccine manufacture: as of now, the system is available for operations of up to 2000 L [125], which is consistent with large-scale production of cell culture-based viral vaccines.

4.8 Conclusions

The present study demonstrated that perfusion-based feeding strategies that minimize media handling and consumption are capable of overcoming metabolic limitations when applied to HCD processes for IAV production. Suspension HEK293SF cultures operated either in fed-batch with continuous harvest or perfusion can be infected at up to 9×10^6 cells/mL without significant loss in *CSVY*, which resulted in an overall increase in the process productivity. We have also demonstrated that the estimation of average uptake/production rates for main metabolites is a useful approach for designing and evaluating feeding strategies, notably during the scaling up of processes, as differences in operation and control of culture parameters can significantly alter cell metabolism. This work also showed that TFDF membranes are suitable for large-scale influenza virus production as the system is scalable, and no virus retention nor loss was observed during the production and harvest steps. In order to validate the production process, further characterization

of the produced virus, as well as the evaluation of the efficacy of the vaccine preparation, should be performed. As HEK293 cells have been shown to be a safe and well-established cell platform, the strategies and processes developed in this study could possibly be applied to intensify the production of other viral vaccines and vectors using this cell substrate. Future work will explore the relation between specific uptake/production rates and online signals, such as capacitance, fluorescence, and specific respiration rate, for online assessment of the metabolic state of cells.

4.9 Supplementary Materials

Supplementary material is presented in appendix A.

4.10 Acknowledgements

The authors acknowledge Repligen Corp for lending the TFDF system and supplying consumables, as well as providing technical support throughout the bioreactor operations.

4.11 Additional information

A schematic representation of the dynamic fed-batch feeding strategy used in this study is presented in Figure 4.8. First, cultures were sampled for determination of VCD and concentration of main nutrients and metabolites. Based on the data obtained, the growth rate and the cell specific consumption/production rates for nutrient/metabolites was calculated for the interval between the current and the previous sample point. Based on the calculated rates, a prediction of nutrient consumption (glutamine, in this study) for the next time interval was made, and the volume of concentrated feed required to replenish the consumed nutrient was calculated.

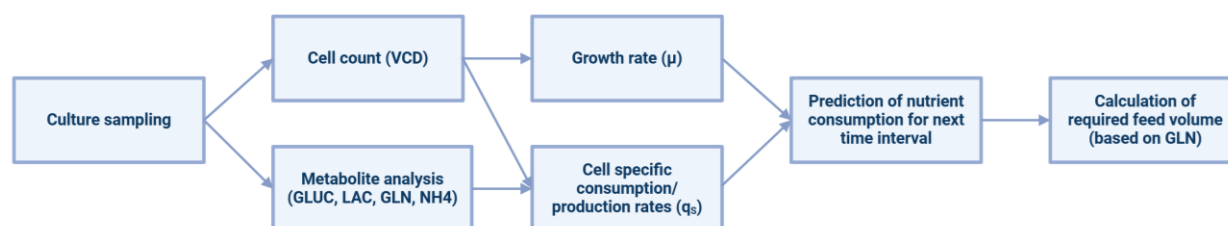


Figure 4.8 Representation of the dynamic fed-batch feeding strategy used. Steps for calculating the volume of concentrated feed to be added during the fed-batch phase of cultures operated in hybrid mode. Created with BioRender.com and used under BioRender's Academic License terms.

A representation of the semi-perfusion operation performed in shake tubes is presented in Figure 4.9. After sampling for VCD determination, cultures were centrifuged (3 min, $300 \times g$) to pellet cells. A pre-determined volume of supernatant was removed (from which samples for quantification of virus and metabolites were taken) and replenished with fresh media. Finally, cells were resuspended.

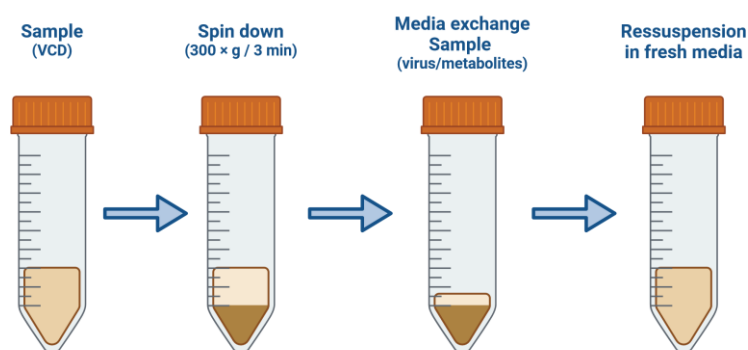


Figure 4.9 Schematic representation of the semi-perfusion operation performed in shake tubes. Created with BioRender.com and used under BioRender's Academic License terms.

A schematic representation of the experimental set-up used for bioreactor cultures operated in hybrid and perfusion mode is presented in Figure 4.10. For perfusion and continuous harvest operations a 30 cm² TFDF module operated by the KML system (Repligen) was employed as cell retention device. Recirculation of the culture broth through the TFDF membrane was maintained at 1 L/min. During fed-batch steps, bolus addition of concentrated feed was performed using a peristaltic pump. During perfusion and continuous harvest steps, permeate flow rate was manually adjusted to correspond to the pre-determined PR. Fresh media was automatically and continuously added based on a weight control system to maintain bioreactor level. Bioreactor sampling was performed every approximately 12 hours for *at line* quantification of cell growth and concentrations of nutrients/metabolites.

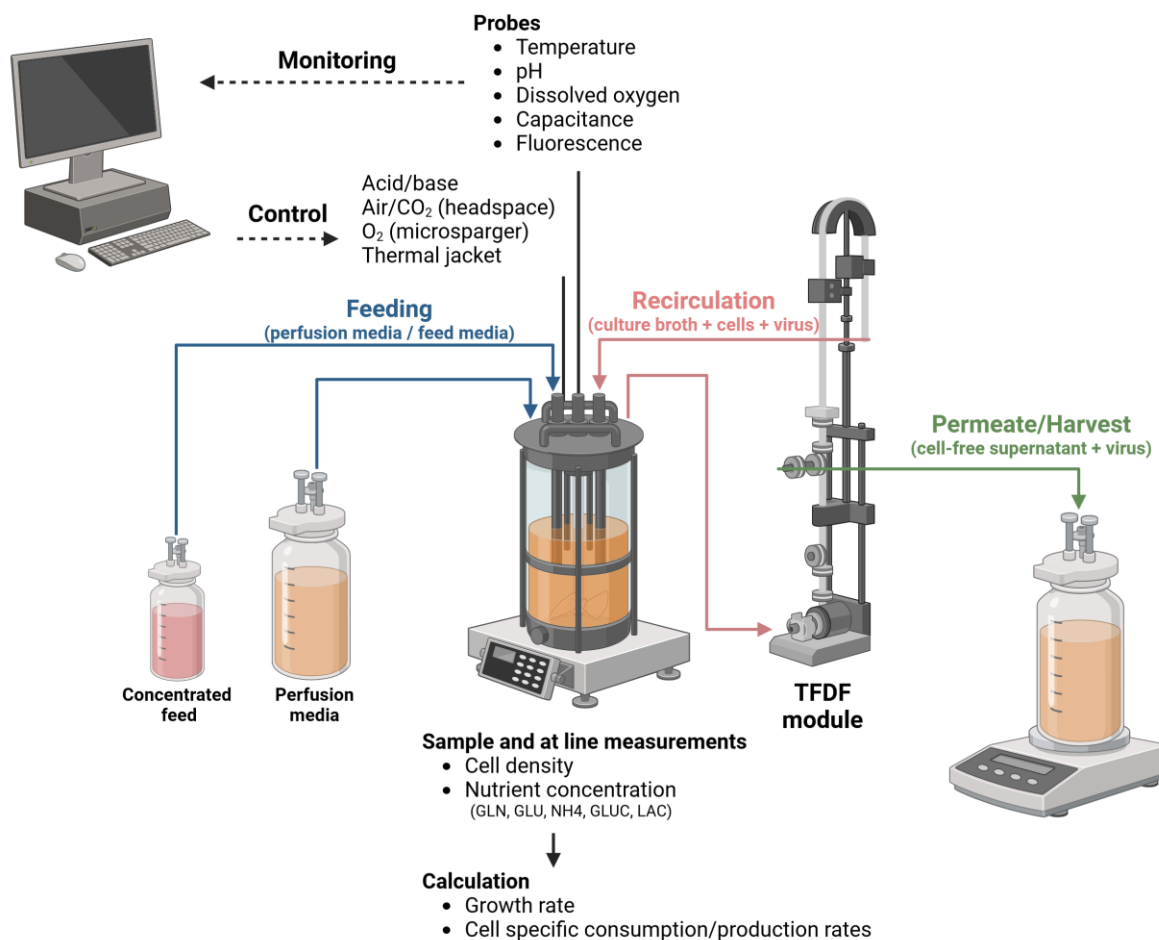


Figure 4.10 Schematic representation of the experimental set-up employed for bioreactor runs operated in hybrid and perfusion mode using a TFDF module as cell retention device. Created with BioRender.com and used under BioRender's Academic License terms.

A representation of the differences between a conventional TFF and a TFDF membrane is presented in Figure 4.11. In conventional TFF modules, larger particles are retained on the surface of the membrane and progressive membrane clogging is often reported for high cell density processes, resulting in the retention of viruses inside of the bioreactor [27, 38, 126]. By combining the tangential flow from conventional TFF processes with a thick wall depth filtration membrane, TFDF modules allow for continuous virus harvesting while avoiding premature filter fouling.

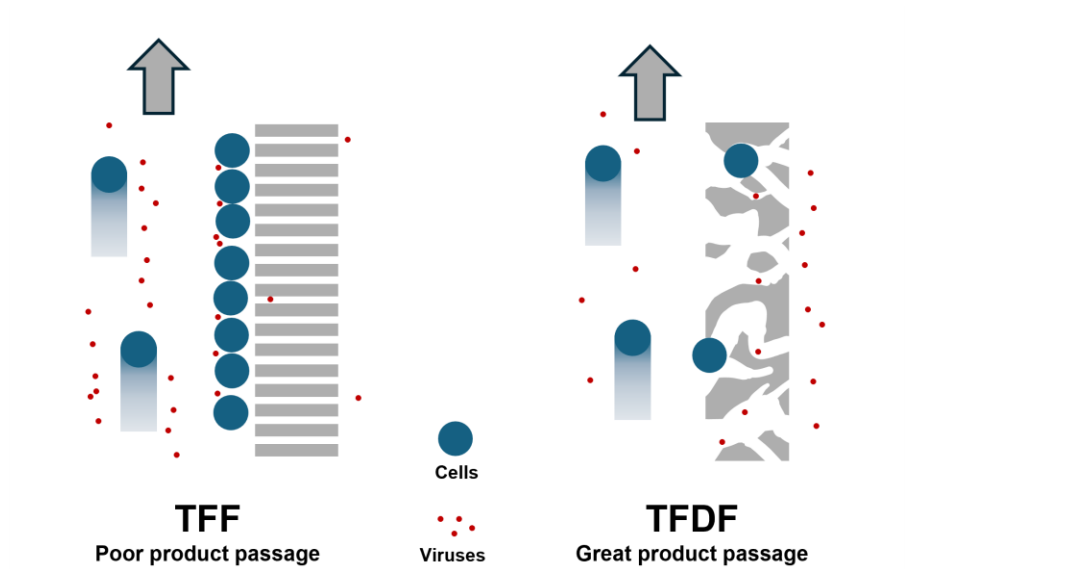


Figure 4.11 Schematic representation comparing a conventional TFF hollow fiber membrane and a TFDF membrane regarding cell retention and virus passage.

4.12 Key points

In this article, we have developed and presented two high cell density processes for influenza virus production in HEK293SF cells. The processes were developed based on feeding strategies that minimized medium consumption and handling.

Small-scale experiments suggested the importance of glutamine supply for virus production and a possible link between glutamine metabolism and the cell density effect.

Feeding strategies were developed based on glutamine consumption, while assuming a similar consumption rate for non-quantified nutrients. The developed processes consisted of:

- A hybrid fed-batch/perfusion: during the growth phase, addition of concentrated feed was performed based on cell growth and specific consumption of glutamine; after infection, the operation was shifted to perfusion to alleviate by-product accumulation and continuously harvest produced viruses.
- A low PR perfusion: during growth and infection phase, cultures were operated in perfusion mode with a PR of 0.5 VVD. To guarantee the supply of non-quantified nutrients, perfusion medium was supplemented with a percentage of concentrated feed.

High cell density cultures operated with the developed strategies presented a metabolic profile comparable to that of cells in a low cell density batch reference.

The dynamic feeding strategies were effective to accommodate the variations in metabolism that occurred during the scale-up of the processes to bench-top bioreactors. No cell density effect was observed for bioreactor cultures infected at around 9×10^6 cells/mL, resulting in up-to 4-fold increase in total virus production when compared to the batch bioreactor reference.

Due to the lower medium consumption, a 1.7-fold increase in the Y/M was observed for the hybrid fed-batch strategy in comparison to the batch reference. The same was not true for the perfusion strategy that, due to higher medium consumption, resulted in a 1.5-fold decrease in Y/M for the same base of comparison.

No virus retention was observed when using a TFDF membrane as cell retention device, both during the infection phase and the final harvest step. TFDF modules would therefore be suitable for integrated/continuous processes for influenza virus production.

CHAPTER 5 ARTICLE 3 : FED-BATCH AND PERFUSION STRATEGIES FOR PROCESS INTENSIFICATION OF MDCK CELL- BASED INFLUENZA VACCINE PRODUCTION

5.1 Article presentation

The article presented in this chapter was submitted for publication to the Journal of Biotechnology on June 11, 2024. Here, we apply the strategies that were developed for influenza virus production in HEK293 cells to produce the same virus using a different cell substrate. While employing an established suspension MDCK cell line, we have further explored fed-batch, hybrid fed-batch/perfusion and perfusion processes for IAV production in high cell density cultures. As MDCK cells have long been identified as the preferred host for influenza virus production, the development of efficient processes with this cell line is critical to consolidate cell culture-based influenza vaccine manufacturing.

First, the basic metabolic profile of cells was characterized in previously established reference cultures performed in shake flasks. Based on this information, a dynamic fed-batch strategy was applied for IAV production in shake-flask cultures operated in fed-batch and hybrid fed-batch/perfusion modes. The best performing hybrid process developed was then scaled up to a 3 L STR bioreactor. Finally, we have evaluated the use of a TFDF membrane for continuous virus harvesting in bioreactor cultures operated in perfusion and hybrid fed-batch/perfusion modes, respectively infected at 15×10^6 cells/mL and 24×10^6 cells/mL.

5.2 Fed-Batch and Perfusion Strategies for Process Intensification of MDCK Cell-Based Influenza Vaccine Production

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5.3 Abstract

Cell culture processes have been increasingly identified as a more flexible and responsive platform for influenza vaccine manufacturing when compared to traditional egg-based systems. In this study, we explored fed-batch and perfusion strategies to intensify influenza A virus (IAV) production in high cell density cultures of suspension Madin-Darby canine kidney (MDCK) cells. To limit the accumulation of toxic by-products in fed-batch operations, a dynamic feeding strategy was developed. A hybrid fed-batch/perfusion process using a tangential flow depth filtration (TFDF) membrane for cell retention allowed for successful IAV production in a culture infected at 15×10^6 cells/mL, resulting in more than 8-fold increase in total production and a 1.5-fold increase in volumetric productivity (VP) when compared to a batch reference. A perfusion process performed using the same cell retention device supported IAV production at 24×10^6 cells/mL, resulting in a more than 15-fold increase in total production and similar VP when compared to the same batch reference. The use of TFDF membranes for cell retention and virus harvest allowed for continuous virus harvesting and clarification in both bioreactor runs, and no loss nor retention of virus was observed during production. Overall, this work provides two options for successful intensification of IAV production in suspension MDCK cells, expanding the arsenal for large-scale cell culture-based vaccine manufacturing.

Keywords: influenza vaccine, perfusion, fed-batch, suspension cell culture, tangential flow depth filtration

Abbreviations: MDCK, Madin-Darby canine kidney; TFDF, tangential flow depth filtration; CSVY, cell specific virus yield; VCD, viable cell density; IAV, influenza A virus; GLN, glutamine; NH₄, ammonium; GLUC, glucose; LAC, lactate; TOI, time of infection; TOH, time of harvest; MOI, multiplicity of infection; B, batch; FB, fed-batch; P, perfusion; FB/P, hybrid fed-batch/perfusion; BR, bioreactor; MD, medium dilution; ME, medium exchange; PR, perfusion rate; CSPR, cell specific perfusion rate; RV, reactor volume; VVD, vessel volume per day; STR, stirred

tank reactor; DO, dissolved oxygen; Y_T , total yield; STY, space time yield; VP, volumetric productivity; TMP, transmembrane pressure.

5.4 Introduction

Worldwide seasonal epidemics of influenza viruses cause up-to 650 000 deaths annually [1], and the high mutation rate of these viruses still poses a constant threat for a global pandemic possibly resulting from an emerging highly infectious variant [3, 49]. While the majority of influenza vaccines are still produced using embryonated hen's eggs [6], cell culture processes have been increasingly demonstrated as a more flexible and responsive platform for vaccine production [7-10]. Moreover, cell culture processes are easily scalable, notably when employing suspension cell lines [127], and advances in equipment and techniques currently allow for operations of 4 000 L even in single-use bioreactors [104]. Madin-Darby canine kidney (MDCK) cells have been identified and suggested as a suitable substrate for cell culture-based influenza vaccine production since the mid-1990s [128], with a high potential to substitute egg-based systems. Although originally adherent, MDCK cells adapted for growth in suspension have been shown to achieve virus titers comparable to non-adapted cells [68], and to support growth to high cell densities of up to 50×10^6 cells/mL in perfusion operations [38]. Like other continuous cell lines, suspension MDCK cells have a tumorigenic potential [82, 129], and the risks arising from its use for vaccine manufacturing must be evaluated and controlled. By using multiple redundant purification processes during influenza vaccine production using a suspension MDCK cell line, Onions, et al. [130] reported reduction of risks to effectively zero by ensuring the absence of cells or oncogenic viruses and decreasing the residual DNA content in the final product. Currently, two cell culture-based vaccines produced in suspension MDCK cells are approved for use: Flucelvax®/Optaflu® (Seqirus) – the first of this type in the market, approved both by the U.S. Food and Drug Administration and European Medicines Agency [9, 131] – and SKYCellflu (SK Bioscience), approved by the South Korean Ministry of Food and Drug Safety [8, 10, 132].

Over the last decades, cell culture processes have greatly evolved to keep up with the high demand for recombinant protein production [21, 40, 103, 133]. While the technologies developed for the latter – such as intensified fed-batch and perfusion, and integrated continuous processing – can in principle be applied for cell culture-based virus manufacturing, the transient nature of most virus production processes, as well as characteristics of the product such as size and instability, makes it

necessary to adapt these strategies for virus production. In that scenario, the development of strategies for cost-effective virus production processes remains a challenge.

Perfusion operations typically allow for higher productivities in a reduced footprint by increasing the cell density and the product titer [18, 21]. This type of operation also enables integrated processing of the produced material [35], which can be critical depending on product stability. As perfusion processes are more complex, requiring the use of a cell retention device and increasing medium consumption and costs, a significant increase in production, when compared to a standard batch culture, is usually pursued [21]. Successful production of influenza virus in perfusion operations using different cell retention devices is reported [25-27, 34, 38, 76, 115], with membrane-based systems being usually favored due to better scalability. However, virus retention due to membrane clogging has been reported for different pore sizes [27, 38]. Tangential flow depth filtration (TFDF) membranes, on the other hand, allow for continuous product recovery throughout the production phase and harvest [32, 33, 126, 134] and have the potential to combine harvest and clarification in a single step [33], making this type of operation compatible with processes that integrate upstream and downstream processing. Such processes are particularly interesting for the production of viruses, where fast product degradation is still a challenge.

Fed-batch operations, on the other hand, are relatively simple, use up lower volumes of medium, and are well established in the biopharmaceutical industry [39]. However, the accumulation of toxic by-products limits the maximum cell densities achieved and, consequently, results in lower titers when compared to perfusion [21]. This is especially detrimental for virus production processes, where by-product accumulation – notably ammonium and lactate – has been linked to reductions in cell specific virus production for different cell lines and viruses, including influenza [34, 134-137]. Moreover, the development of culture medium and feeds specifically tailored for vaccine manufacturing processes and cell lines is limited, and the available feeds – mostly designed for recombinant protein production in CHO cells – might not respond to the needs of virus production processes. Currently, almost all fed-batch processes applied for virus production reported in the literature are still limited to cultivations at relatively low cell concentrations [138-140]. Nevertheless, intensified fed-batch operations – inoculated at higher cell densities – and advances in the design and control of feeding strategies allow one to reach higher cell concentrations while limiting by-product accumulation [15, 17, 40, 103, 114, 140], and could be applied for virus production processes. Besides, other feeding strategies that combine fed-batch

and perfusion have also shown potential for virus production [29]. In a previous work, a hybrid fed-batch/perfusion process with continuous harvesting was developed for influenza production in cultures of HEK293 cells infected at 10×10^6 cells/mL with no loss in cell specific virus yield (CSVY) when compared to a batch reference [134]. Although more complex than a traditional fed-batch, hybrid processes alleviate by-product accumulation after infection, therefore reducing the impact of high cell concentrations in virus production and allowing for relatively higher cell densities while maintaining medium consumption at low levels. Additionally, depending on the type of cell retention device employed, this type of process allows for the continuous harvesting of the produced virus, and therefore could be applied when integrated processes are preferable.

In this study, we explored different fed-batch-based strategies for influenza A virus (IAV) production in high cell density cultures of MDCK cells. The different feeding regimens were developed based on cell specific consumption rates of glucose and glutamine to supply for critical nutrients while limiting by-product accumulation. Furthermore, we evaluated the use of a TFDF system as cell retention and virus harvest device for continuous harvesting of IAV in 3 L STR bioreactors operated in perfusion and hybrid fed-batch/perfusion.

5.5 Materials and Methods

5.5.1 Cell line and culture conditions

Suspension MDCK.Xeno cells, previously adapted from adherent MDCK cells (ECACC, #84121903) to SMIF8 medium [141] and further adapted to single cell suspension [123], were maintained in XenoTM basal medium (Shanghai BioEngine Sci-Tech). Basal medium was supplemented with 6 mM L-glutamine for cell maintenance while lower glutamine set points were used for the feeding strategies developed latter on (Table 1). Cells were cultured in 125 mL polycarbonate baffled shake flasks (35 mL working volume, Corning) in a Multitron Pro orbital shaker (50 mm shaking orbit; Infors HT) at 185 rpm, 37°C, and 5% CO₂. Viable cell density (VCD) and viability was determined using a Vi-CellTM XR Cell Viability analyzer (Beckman Coulter). Quantification of main metabolites, glutamine (GLN), ammonium (NH₄), glucose (GLUC) and lactate (LAC), was performed using a Cedex Bio[®] Analyzer (Roche). Cell specific consumption/production rates for main metabolites (q_{GLN} , q_{NH_4} , q_{GLUC} and q_{LAC} , respectively for glutamine, ammonium, glucose and lactate), as well as molar lactate-to-glucose ratio ($Y_{LAC/GLUC}$) and ammonium-to-glutamine ratio ($Y_{NH_4/GLN}$) were calculated as described in Silva, et al. [134].

To limit variability between the different experiments, all of the cultures described here were performed using cells from the same cell bank at the same passage number range (between 5 and 15 passages).

5.5.2 Virus and infection conditions

Virus seed stock for influenza A/Puerto-Rico/8/34 (H1N1), originally provided by the Robert Koch institute (Amp. 3138) and subsequently adapted to MDCK.Xeno cells, was produced in MDCK.Xeno cells (7.7×10^8 TCID₅₀/mL). For all cultures described in this study, infections were performed with the addition of trypsin (5000 U/mL in PBS, Thermo Fisher Scientific, #27250–018) directly to the culture at time of infection (TOI) to a final concentration of 20 U/mL. The multiplicity of infection (MOI), calculated based on tissue culture infectious dose titer (TCID₅₀/mL), varied between 0.1 and 0.001 (Table 5.1). At TOI, a temperature shift to 32°C was performed, as a positive effect of temperature reduction in the production of influenza virus was previously reported [38]. Samples for virus quantification were collected, centrifuged ($3000 \times g$, 10 min, 4°C), aliquoted, and stored at -80°C until further analysis.

5.5.3 Feeding strategies and modes of operation

Cultures were operated in batch (B), hybrid fed-batch/perfusion (FB/P), perfusion (P), and fed-batch (FB) modes, the latter performed only in shake flasks, and the three first performed both in shake flasks and bioreactor with feeding strategies applied essentially in the same manner for both scales. FB/P cultures were defined by a fed-batch operation during the growth phase and a perfusion operation after infection. The experimental set-up for shake flask and bioreactor cultures is presented in the following sections. A detailed description of the feeding strategies tested is presented in Table 5.1. Infections were performed as described above.

Batch cultures were seeded at 0.8×10^6 cells/mL in fresh medium and cultivated to $3.5\text{--}4.5 \times 10^6$ cells/mL, followed by a 1:2 dilution step with fresh medium (MD) to around 2×10^6 cells/mL at TOI, as described by Pelz, et al. [34].

For the design of the fed-batch strategies, latter applied to cultures operated in FB/P and FB modes, preliminary experiments were conducted to evaluate two concentrated, chemically defined and serum-free feeds, Cell BoostTM 5 (Cytiva) and HEK FS (Xell-Sartorius). Briefly, non-infected cultures were spiked with varying volumes of feed, while osmolarity, cell growth, and nutrient

consumption were assessed (data not shown). As the addition of Cell Boost™ 5 resulted in increased ammonium formation and lower cell growth, all fed-batch-based strategies were developed using the HEK FS feed. During the growth phase of cultures operated in FB and FB/P, daily addition of HEK FS feed supplemented with 50 mM glutamine was performed at 12 h intervals. As preliminary experiments suggested that the control of glutamine concentration, a limiting nutrient, was critical to reduce ammonium formation, the addition of feed was performed based on glutamine while assuming that other critical non-quantified nutrients were consumed at a similar rate. A dynamic feeding strategy was adopted: for each culture, the volume of concentrated feed to be added at a given interval time was determined based on the average growth rate and cell specific glutamine consumption rate for the previous interval, calculated as described by Silva, et al. [134]. Based on those values and using mass balance equations, a prediction of glutamine consumption for the next interval was made, and a corresponding volume of concentrated medium was added to maintain glutamine concentration above a given fixed setpoint (Table 5.1). The total volume of concentrated feed added varied slightly depending on the condition but remained below 15% of the final culture volume for shake flask and bioreactor experiments. To accommodate the addition of feed medium, FB and FB/P cultures were started with 85% of the final working volume. The concentration of glutamine at inoculation varied depending on the feeding strategy to limit initial accumulation of ammonium (Table 5.1). After infection of cultures operated in FB/P, a perfusion step at a pre-defined perfusion rate (PR) was started (performed semi-continuously for shake flask cultures and continuously through a TFDF membrane for bioreactors). FB cultures, on the other hand, had further concentrated feed additions or were submitted to a MD step at TOI.

The semi-perfusion and perfusion operations employed in this study were performed as previously described [115, 123]. A cell specific perfusion rate (CSPR) of 100 pL/cell/day for the shake flask experiments and of 160 pL/cell/day for the bioreactor culture was used, which was defined based on the nutrient requirements of the cell line. A higher CSPR was defined for the bioreactor culture as cells had shown higher consumption of nutrients when cultured in the bioreactor. Moreover, as the semi-continuous exchange allows for a more effective medium usage in shake flasks, a higher CSPR is required to maintain the same nutrient and by-product levels in bioreactor cultures. For the perfusion bioreactor operation, the permeate flow rate was manually adjusted every 12 h to correspond to the pre-defined CSPR, following a profile determined based on the average growth

rate, as described in Hein, et al. [115]. For shake flasks, the volume of medium to be exchanged for a given interval (V_E) at a given time (t_n) was calculated based on the following equation :

$$V_E = \frac{X_n}{\mu} * (e^{\mu * \Delta t} - 1) * V_R * CSPR \quad (5.1)$$

where X_n is the current VCD, μ is the average growth rate calculated for the previous interval, Δt ($t_{n+1}-t_n$) is the interval until the next feeding time, and V_R in the current culture volume.

Table 5.1 Summary of different feeding regimes evaluated for IAV production in suspension MDCK.Xeno cells.

Condition code	V _w (mL)	GLN ₀ (mM)	VCD ₀ (10 ⁶ cells/mL)	VCD _{TOI} (10 ⁶ cells/mL)	Growth phase	TOI	MOI	Infection phase
B-1 ^a	35	8.0	0.8	2 (4→2) ^c	batch	MD	0.001	batch
P-1 ^a	35	8.0	1.2	25	semi-perfusion; 100 pL/cell/day	ME	0.001	semi-perfusion; 2 VVD
FB/P-1 ^a	35	6.0	1.5	10	fed-batch; GLN > 2.0 mM	ME	0.001	semi-perfusion; 1 VVD
FB/P-2 ^a	35	6.0	1.5	14	fed-batch; GLN > 2.0 mM	ME	0.001	semi-perfusion; 1.2 VVD
FB/P-3 ^a	35	4.0	1.5	15	fed-batch; GLN > 0.5 mM	ME	0.001	semi-perfusion; 1.2 VVD
FB/P-4 ^a	35	4.0	1.5	15	fed-batch; GLN > 0.5 mM	ME	0.001	semi-perfusion; 1.2 VVD; supp. medium ^d
FB/P-5 ^a	35	2.5	4.0	20	fed-batch; GLN > 0.2 mM	ME	0.001	semi-perfusion; 2 VVD
FB-1 ^a	35	4.0	1.0	5 (10→5) ^c	fed-batch; GLN > 0.5 mM	MD	0.1	batch
FB-2 ^a	35	2.5	4.0	10 (20→10) ^c	fed-batch; GLN > 0.2 mM	MD	0.1	batch
FB-3 ^a	35	2.5	4.0	20	fed-batch; GLN > 0.2 mM	N/A	0.1	fed-batch; GLN > 0.2 mM
BR-B ^b	700	8.0	0.8	2 (4→2) ^c	batch	MD	0.001	batch
BR-P ^b	1250	8.0	1.2	24	perfusion (TFDF); 160 pL/cell/day	ME (1 RV)	0.001	perfusion (TFDF); 2.5–3.0 VVD
BR-FB/P ^b	1250	4.0	1.5	15	fed-batch; GLN > 0.5 mM	ME (1 RV)	0.001	perfusion (TFDF); 1.5–2.0 VVD; supp. medium ^d

^a Single biological replicate.

^b Single biological replicate with quantifications performed in duplicates.

^c Values in the parenthesis indicate the VCD before and after the MD step.

^d Basal medium supplemented with 3.5% (v/v) of concentrated feed medium.

GLN₀, initial glutamine concentration; VCD₀, VCD at inoculation; VCD_{TOI}, VCD at time of infection; RV, reactor volume; B, batch; FB, fed batch; FB/P, hybrid fed-batch/perfusion; P, perfusion; BR, bioreactor; ME, medium exchange; MD, 1:2 dilution with fresh medium; MOI, multiplicity of infection; TOI, time of infection; N/A, not applicable; VVD, vessel volume per day.

5.5.4 Shake flask experimental set-up

Small-scale screening experiments were conducted in 125 mL baffled shake flasks (35 mL working volume), using the conditions described above. After infection, culture flasks were incubated at 32°C for the infection phase. Perfusion operations (cultures operated in P or FB/P, the latter comprising a perfusion step after infection) were carried out semi-continuously in shake flasks: at given intervals, cells were pelleted by centrifugation ($300 \times g$, 5 min) and a pre-determined volume of supernatant (up-to 80% of the working volume) was removed and replenished with fresh medium. As cell concentration increased, shorter intervals between each step were necessary to exchange the total daily volume of medium required, varying from every 12 h to every 8 h. For feeding strategies involving a medium exchange step (ME) before infection, cells were pelleted, and the totality of the supernatant was exchanged for fresh medium.

5.5.5 Bioreactor experimental set-up

Production of IAV in batch was performed in a 1 L STR bioreactor (DASGIP, Eppendorf AG) with a working volume (V_w) of 0.7 L, equipped with a pitched blade impeller (50 mm diameter). For hybrid fed-batch/perfusion and perfusion operations, a 3 L STR bioreactor (DASGIP, Eppendorf AG) with a V_w of 1.3 L, equipped with two pitched blade impellers (50 mm diameter) was used. The bioreactor setups used in this study were previously described and optimized for this cell line [142, 143].

Bioreactors were maintained at 37°C and pH 7.6 until infection, and a shift to 32°C and pH 7.4 was performed at TOI, which was maintained until the end of the run. Dissolved oxygen (DO) was controlled at 50% air saturation by varying gas flow rates (3–9 L/h) and oxygen content (21-100%) flowing through a L-drilled hole macrosparger. For high cell density cultures operated in perfusion or hybrid mode in the 3 L bioreactor, an additional supply of pure oxygen (0.5 L/h) through a macrosparger was manually activated when the primary DO controller reached its maximum capacity (at around 10×10^6 cells/mL). The pH was controlled through the addition of NaHCO_3 (90 g/L) or through the sparging of CO_2 .

The bioreactor operated in hybrid mode was inoculated at 85% of the final working volume to accommodate for the addition of concentrated feed during the growth phase. For perfusion and hybrid operations, a 30 cm² TFDF polypropylene cartridge (pore size 2–5 μm , Repligen) operated by the Krosflo TFDF system (Repligen) was used as cell retention and virus harvesting device. The

recirculation rate was maintained at 1 L/min, measured by an integrated flowmeter. Perfusion medium was added continuously to maintain bioreactor level based on a weight control system, according to the permeate flow rate. As a complete medium exchange was not feasible for the bioreactor runs, one reactor volume (RV) was exchanged right before the infection by increasing the permeate flowrate to 30 mL/min for around 40 min. After the infection, the perfusion was kept off for one hour to allow virus entry into the cells. After infection and during the virus production phase, the permeate was collected in ice-cooled polyethylene terephthalate bottles. At every sample point, the bottle was exchanged and its content (permeate pool) was sampled. At time of harvest, determined based on the viability (cell count) and on the biovolume (capacitance probe, Incyte Hamilton), the bioreactor content was harvested and clarified through the cell retention device. This final harvest consisted of a three-step process, comprising a concentration, a diafiltration (performed with fresh medium), and a second concentration step, which resulted in a 20% increase in the final volume. The volumes of harvested material for each of these steps is presented in Table B1. During the harvest step, the permeate flow rate was maintained at 30 mL/min and the recirculation rate was increased to 2.1 L/min, to avoid membrane clogging and promote self-cleaning.

5.5.6 Virus quantification and productivity evaluation

To quantify virus production, the hemagglutinin titer (HA; $\log_{10}(\text{HAU}/100 \mu\text{L})$), was evaluated by the hemagglutination assay, performed with chicken erythrocytes as described in Kalbfuss, et al. [144]. As an internal standard was employed to correct deviations in measured values due to the varying quality of the erythrocyte preparation, the assay has been validated with a standard deviation of $\pm 0.03 \log_{10}$ (standard error of +15%/–13% for HA activity with a confidence level of 95 %). An estimation of total viral particle titer (C_{virion} ; virions/mL) based on the HA titer – to facilitate comparisons with other studies – was performed based on the following equation:

$$C_{\text{virion}} = 10^{\text{HA}} * C_{\text{RBC}} \quad (5.2)$$

where C_{RBC} is the concentration of red blood cells used for the assay (2×10^7 cells/mL).

The infectious virus titer, corresponding to the concentration of infectious viral particles (IVP) (C_{IVP} ; IVP/mL) was determined by TCID₅₀ assay as described in Genzel and Reichl [145] with a measurement error of $\pm 0.3 \log_{10}$ [146].

The total virus yield (Y_T ; virions, IVP) was calculated with the following equation:

$$Y_T = C_R * V_R + \sum C_P * V_P \quad (5.3)$$

where C_R is the virus titer inside the bioreactor at a given time, C_P is the virus titer in the permeate pool for a given time interval, V_R is the current culture volume, V_P is the permeate pool volume.

The cell specific virus yield, calculated based on the total viral particle (CSVY) and on the infectious titer (CSVY_{IVP}), was determined as follows:

$$CSVY = \frac{Y_{T,virions}}{X_T * V_W} \quad (5.4)$$

$$CSVY_{IVP} = \frac{Y_{T,IVP}}{X_T * V_W} \quad (5.5)$$

where $Y_{T,virions}$ is the total viral particle yield, $Y_{T,IVP}$ is the total infectious viral particle yield, X_T is the maximum total cell density and V_W is the working volume.

Productivity was evaluated based on the volumetric productivity (VP), and the space time yield (STY), calculated as follows:

$$VP = \frac{Y_{T,virions}}{V_T * t_T} \quad (5.6)$$

$$STY = \frac{Y_{T,virions}}{V_W * t_T} \quad (5.7)$$

where V_T is the total volume of medium consumed, and t_T is the total duration of the culture [147].

5.6 Results

5.6.1 Production of IAV in shake flask cultures operated in batch and semi-perfusion mode

To characterize cell growth, nutrient consumption, and metabolite formation in processes for IAV production in suspension MDCK.Xeno cells, two reference cultures were performed in shake flasks. First a low cell density batch, infected at 2×10^6 cells/mL after a dilution step at TOI (B-1),

and second, a high cell density semi-perfusion infected at 25×10^6 cells/mL, with a medium exchange step at TOI (P-1). Cells grew exponentially until infection for both conditions and, as a result of the temperature shift to 32°C, the growth rate drastically decreased after infection. Viability remained above 98% until 24 hpi, followed by a sharp decrease in the following hours (Figure 5.1a). During the growth phase, glutamine concentration remained above 4 mM for B-1 and varied between around 2–7 mM for P-1 due to the semi-continuous exchange of medium (Figure 5.1c), while ammonium concentrations remained below 4 mM for both conditions throughout the process (Figure 5.1d). Glucose and lactate concentrations remained above 30 mM and below 15 mM, respectively, for both conditions (Figure B1). An average doubling time (t_D) of 18 h and 21 h was obtained for conditions B-1 and P-1, respectively, and cell specific consumption/production rates for main metabolites (q_{GLN} , q_{GLUC} , q_{NH4} and q_{LAC}) were more than doubled for the low cell density batch compared to the semi-perfusion culture. Higher molar lactate-to-glucose ($Y_{LAC/GLUC}$) and ammonium-to-glutamine ($Y_{NH4/GLN}$) ratios indicated a less efficient metabolism for the low cell density batch (Table B2). Maximum titers of 3.13 $\log_{10}(\text{HAU}/100 \mu\text{L})$ and 3.92 $\log_{10}(\text{HAU}/100 \mu\text{L})$ were obtained, respectively, for the batch and semi-perfusion cultures, between 30–36 hpi (Figure 5.1e). The higher titer obtained with the semi-perfusion operation translated into a 11-fold increase in Y_T (for the same vessel volume), a 6-fold increase in STY, and similar VP and CSVY, in comparison to the batch reference (Figure 5.1b, Table 5.2).

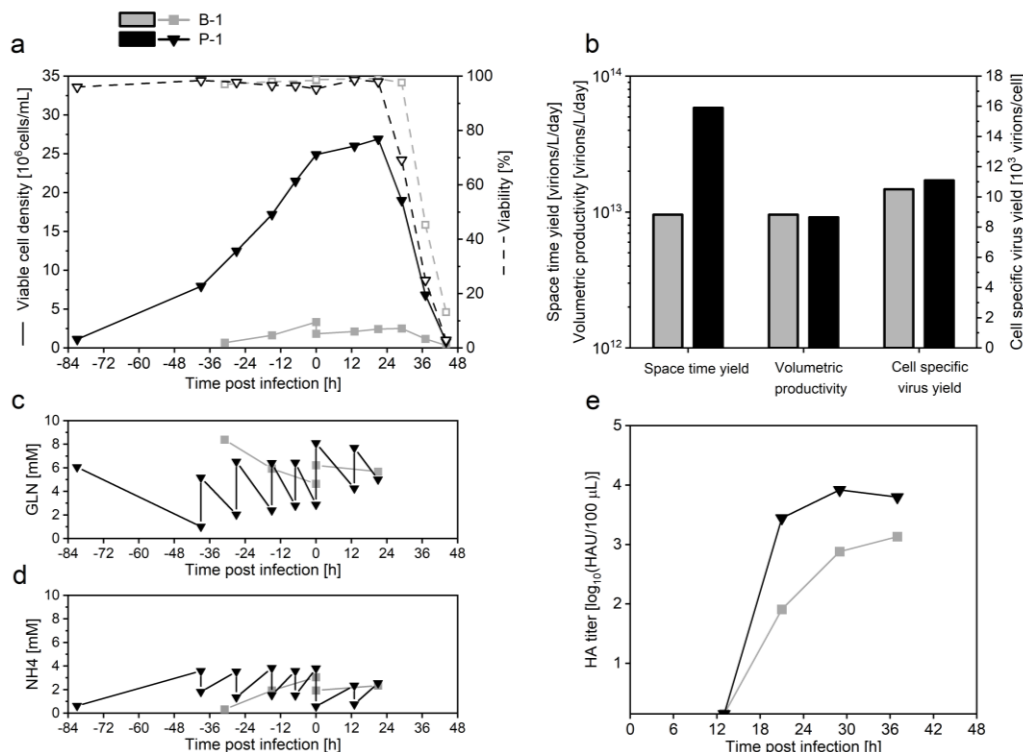


Figure 5.1 IAV production in suspension MDCK.Xeno cells performed in 125 mL baffled shake flasks operated in batch (B-1) and semi-perfusion (P-1). Cells cultivated in batch were grown to around 3.5×10^6 cells/mL and, after a MD step, infected at 2×10^6 cells/mL. For semi-perfusion operation, a determined volume of medium (corresponding to a CSPR of 100 pL/cell/day) was exchanged every 8–12 h, starting at day 2, with infection performed at 25×10^6 cells/mL. Infections were carried out with an MOI of 0.001 with addition of 20 U/mL of trypsin. At TOI, the temperature was reduced to 32°C. **a)** Viable cell density (full) and viability (empty), **b)** productivity, **c)** glutamine concentration, **d)** ammonium concentration, and **e)** virus concentration (HA titer).

5.6.2 Development of hybrid fed-batch/perfusion strategies for IAV production in shake flasks

A first hybrid fed-batch/perfusion strategy was designed and, based on the glutamine profile obtained for the reference cultures (Figure 5.1c), glutamine concentration was kept above 2 mM during the growth phase through the addition of concentrated feed. Cultures were infected at 10×10^6 cells/mL (FB/P-1) and 14×10^6 cells/mL, (FB/P-2). At time of infection, a ME was performed, and cultures were then operated in semi-perfusion. For both conditions tested, glutamine concentration varied around 2–5 mM during the growth phase (Figure 5.2c). An average t_D of 26 h for FB/P-1 and 30 h for FB/P-2 was obtained (Table B2), indicating suboptimal conditions for

cell growth. In fact, ammonium concentrations attained very high levels early on for both conditions, up-to 7.5 mM at TOI for FB/P-2 (Figure 5.2d). Maximum titers of $3.74 \log_{10}(\text{HAU}/100 \mu\text{L})$ were obtained for both conditions at 38 hpi (Figure 5.2e). While the CSVY obtained for both conditions was comparable to the batch reference, the longer process duration due to the higher t_D resulted in only 3-fold increase in STY and up-to 5-fold increase in Y_T in comparison to the batch culture (Figure 5.2b, Table 5.2). In this scenario, the higher cell concentration of FB/P-2 seemed not beneficial, leading to a slight loss of CSVY. No meaningful difference in productivity (STY, VP) between cultures FB/P-1 and FB/P-2 was observed (Figure 5.2b).

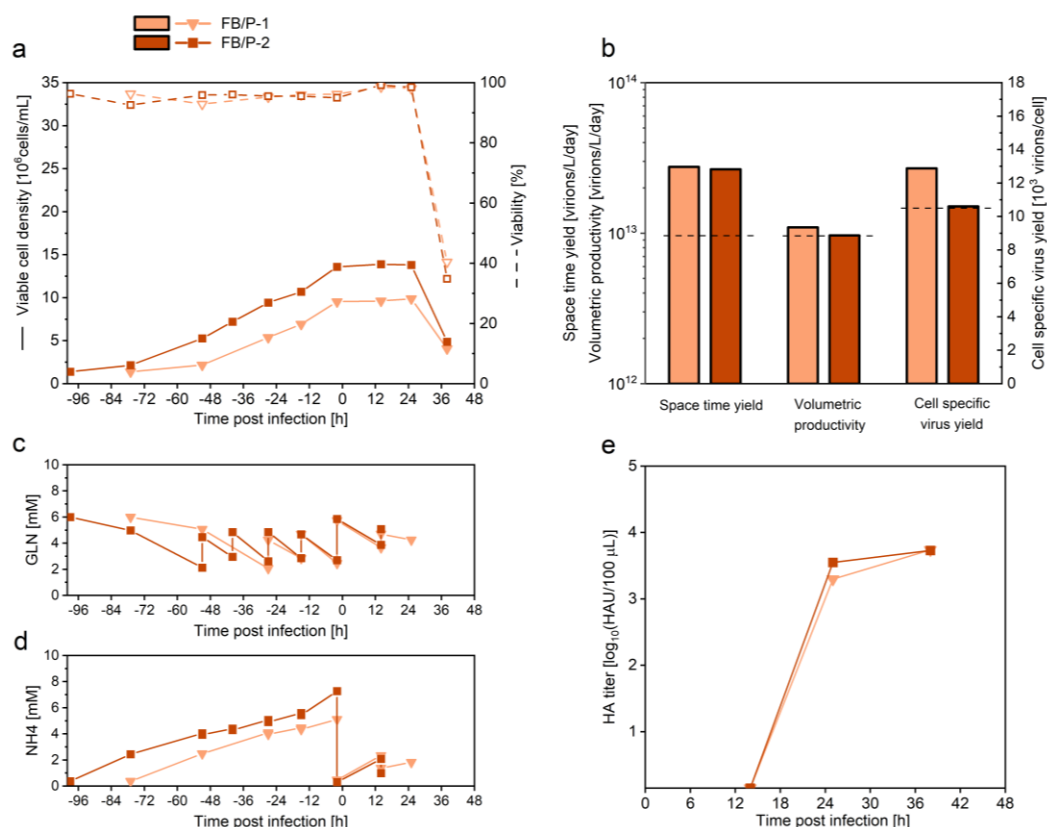


Figure 5.2 IAV production in MDCK.Xeno cells in 125 mL baffled shake flasks operated in FB/P, infected at 10×10^6 cells/mL (FB/P-1) and 14×10^6 cells/mL (FB/P-2). During the growth phase, concentrated feed was added to maintain glutamine concentration above 2 mM. After infection, performed following a ME step, a determined volume of medium (corresponding to a PR of 1 VVD and 1.2 VVD for FB/P-1 and FB/P-2, respectively) was exchanged semi-continuously every 8–12 h. Infections were performed with an MOI of 0.001 with addition of 20 U/mL of trypsin. At TOI, the temperature was reduced to 32°C. **a**) Viable cell density (full) and viability (empty), **b**) productivity (dashed lines indicate values for the B-1 reference condition), **c**) glutamine concentration, **d**) ammonium concentration, and **e**) virus concentration (HA titer).

Three other hybrid strategies were then designed aiming to limit ammonium accumulation by decreasing glutamine supply. Addition of concentrated feed to the cultures was made to maintain glutamine concentration above 0.5 mM during the growth phase for conditions FB/P-3 and FB/P-4, both infected at 15×10^6 cells/mL. For the latter, perfusion medium (used after infection during the continuous harvesting step) was supplemented with 3.5% of concentrated feed (Table 5.1). To evaluate if a hybrid operation could sustain infections at a higher cell density, culture FB/P-5, infected at 20×10^6 cells/mL, had concentrated feed added to maintain glutamine concentration above 0.2 mM. Moreover, the culture was seeded at a higher VCD (4×10^6 cells/mL), as previous experiments had shown that this resulted in lower cell specific production of ammonium in the first 24 h of culture. Glutamine concentration varied between 0.6–3 mM for FB/P-3 and FB/P-4, and 0.5–2.5 mM for FB/P-5 (Figure 5.3c), which resulted in maximum ammonium concentrations at TOI of 4.7 mM for the first two strategies and 3.4 mM for the latter (Figure 5.3d). During the growth phase, an average t_D of 22 h was obtained for cultures FB/P-3 and FB/P-4, and of 21 h for culture FB/P-5 (Table B2). For cultures infected at 15×10^6 cells/mL, maximum HA titers were reached at 36 hpi (Figure 5.3e), respectively $3.81 \log_{10}(\text{HAU}/100 \mu\text{L})$ and $3.88 \log_{10}(\text{HAU}/100 \mu\text{L})$ for conditions FB/P-3 and FB/P-4. The comparable CSVY and the shorter process duration due to low t_D resulted, in comparison to the batch reference and respectively for FB/P-3 and FB/P-4, in 6.4-fold and 7.3-fold increase in Y_T , and 4-fold and 4.6-fold increase in STY (Figure 5.3b, Table 5.2). Overall, this represented a more pronounced improvement in relation to the batch reference when compared to the first FB/P-2 strategy. Although ammonium concentration was below 4 mM throughout the process for the FB/P-5 culture, a drop in CSVY in comparison to the batch reference was observed. This resulted in a decrease in titer (maximum HA titer of $3.75 \log_{10}(\text{HAU}/100 \mu\text{L})$ for condition FB/P-5) and no meaningful difference in productivity between the cultures infected at 15×10^6 cells/mL and 20×10^6 cells/mL operated in hybrid mode (Figure 5.3b, Figure 5.3e). Because of the lower medium consumption, an increase of up-to 1.5-fold in VP was observed for the FB/P conditions in comparison to the batch and semi-perfusion reference cultures.

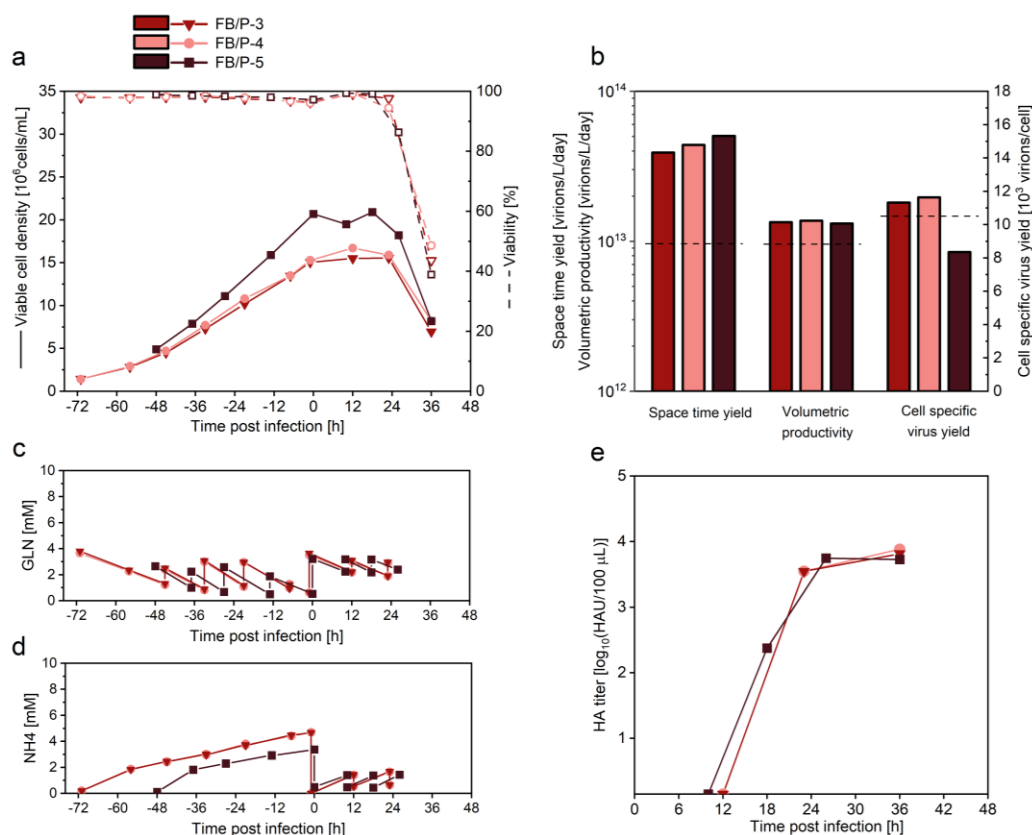


Figure 5.3 IAV production in MDCK.Xeno cells in 125 mL baffled shake flasks operated in FB/P, infected at 15×10^6 cells/mL (FB/P-3), 15×10^6 cells/mL with supplemented perfusion medium (FB/P-4), and 20×10^6 cells/mL (FB/P-5). During the growth phase, concentrated feed was added to maintain glutamine concentration above 0.5 mM (FB/P-3 and FB/P-4) and above 0.2 mM (FB/P-5). After infection, performed following a ME step, a determined volume of medium (corresponding to a PR of 1.5 VVD for FB/P-3 and FB/CH-4, and 2 VVD for FB/P-5) was exchanged every 8–12 h. Infections were performed with an MOI of 0.001 with addition of 20 U/mL of trypsin. At TOI, the temperature was reduced to 32°C. **a**) Viable cell density (full) and viability (empty), **b**) productivity (dashed lines indicate values for the B-1 reference condition), **c**) glutamine concentration, **d**) ammonium concentration, and **e**) virus concentration (HA titer).

5.6.3 Evaluation of fed-batch strategies for IAV production in shake flasks

To assess whether fed-batch operations, without a ME step or continuous harvesting of virus, could be applied for IAV production in suspension MDCK cells, three conditions were tested. First, the culture FB-1 was cultivated to 10×10^6 cells/mL, followed by a dilution step at TOI and infection performed at 5×10^6 cells/mL. Second, the culture FB-2 was cultivated to 20×10^6 cells/mL, followed by a dilution step at TOI and infection performed at 10×10^6 cells/mL, and third, culture FB-3 was cultivated until 20×10^6 cells/mL, with infection performed without a MD step (Figure

5.4a). For all three conditions, low levels of glutamine were maintained to limit ammonium formation during the growth phase (Figure 5.4c). The infection was performed using a MOI of 0.1 – a 100-fold increase compared to previously described conditions, as initial experiments had shown that the higher MOI had no impact on maximum titer but resulted in peak production up to 10 hours earlier (Figure B6) – to shorten the infection phase and reduce ammonium accumulation after infection. The dynamic feeding strategies were able to maintain ammonium concentration below 4 mM until the end of the production phase for all three conditions (Figure 5.4d). While culture FB-1 showed a comparable CSVY to the batch reference, cultures FB-2 and FB-3 showed up-to 50% reduction in CSVY (Figure 5.4b), even though ammonium concentrations remained below 4 mM indicating that some kind of limitation or inhibition occurred, which could be related to the depletion of non-quantified nutrients or accumulation of non-quantified metabolites. Maximum HA titers of $3.51 \log_{10}(\text{HAU}/100 \mu\text{L})$, $3.64 \log_{10}(\text{HAU}/100 \mu\text{L})$ and $3.76 \log_{10}(\text{HAU}/100 \mu\text{L})$ were obtained respectively for conditions FB-1, FB-2 and FB-3 (Figure 5.4e). The fed-batch operations resulted, when compared to the batch reference, in an increase in Y_T of 3.1-fold, 3.4-fold, and 4.6-fold and an increase in VP of 2.4-fold, 2.6-fold and 3.4-fold, respectively for FB-1, FB-2 and FB-3.

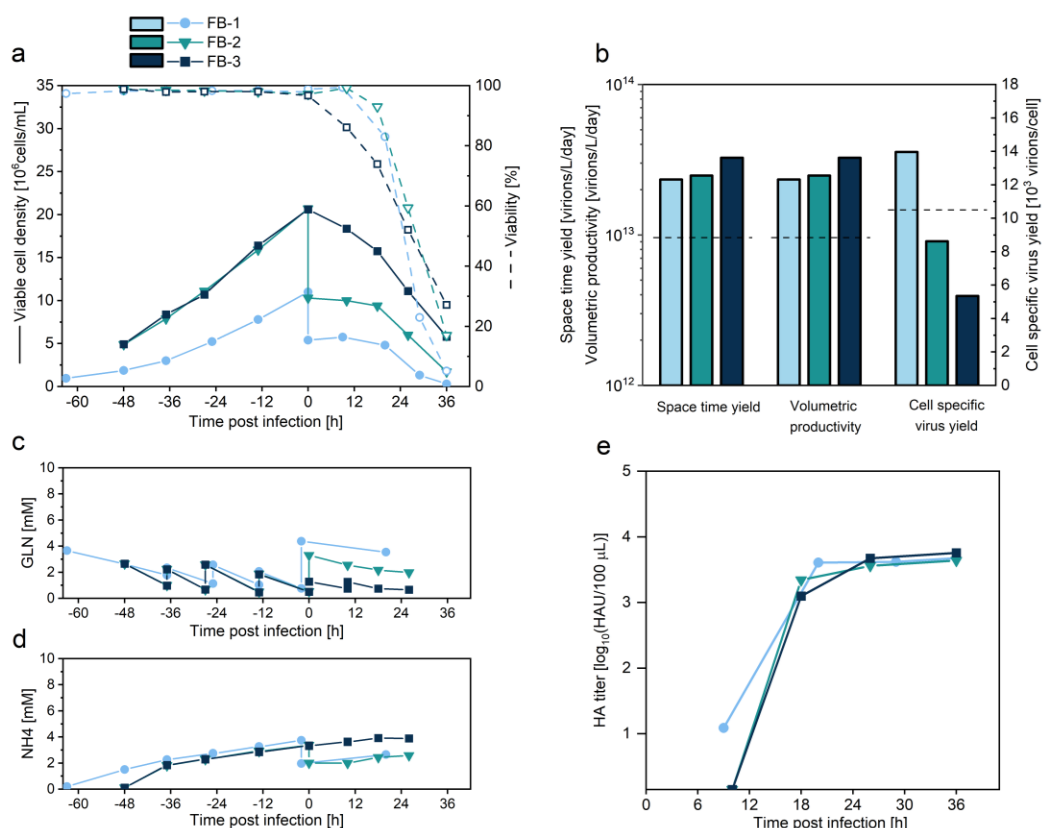


Figure 5.4 IAV production in MDCK.Xeno cells in 125 mL baffled shake flasks operated in fed-batch. Concentrated feed was added to maintain glutamine concentration above 0.2 mM. Cultures were infected at 5×10^6 cells/mL after a MD step (FB-1), 10×10^6 cells/mL after a MD step (FB-2), and 20×10^6 cells/mL (FB-3). Infections were performed with an MOI of 0.1 with addition of 20 U/mL of trypsin. At TOI, the temperature was reduced to 32°C. **a)** Viable cell density (full) and viability (empty), **b)** productivity (dashed lines indicate values for the B-1 reference condition), **c)** glutamine concentration, **d)** ammonium concentration, and **e)** virus concentration (HA titer).

Table 5.2 Summary of results for IAV production in shake flask and bioreactor cultures of suspension MDCK.Xeno cells.

Code	VCD _{MAX} [10 ⁶ cells/mL]	V _T	V _{Feed}	HA _{MAX} [log ₁₀ (HAU/100 µL)]	CSVY [10 ³ virions/cell]	CSVY _{IVP} [IVP/cell]	Y _T ^{a,b} [virions]	STY ^b [virions/L/day]	VP ^b [virions/L/day]
B-1	3.8	1.0 × V _W	N/A	3.13	10.5	N/D	9.5 × 10 ¹¹ (reference)	9.6 × 10 ¹² (reference)	9.6 × 10 ¹² (reference)
P-1	26.9	7.1 × V _W	N/A	3.92	11.1	N/D	11.1-fold	6.0-fold	similar
FB/P-1	10.1	2.9 × V _W	0.08 × V _W	3.74	12.9	N/D	4.4-fold	3.0-fold	similar
FB/P-2	13.9	3.2 × V _W	0.15 × V _W	3.74	10.6	N/D	5.2-fold	2.8-fold	similar
FB/P-3	15.7	3.2 × V _W	0.10 × V _W	3.81	10.9	N/D	6.4-fold	4.1-fold	1.3-fold
FB/P-4	16.7	3.2 × V _W	0.10 × V _W	3.88	11.6	N/D	7.3-fold	4.6-fold	1.5-fold
FB/P-5	20.9	3.8 × V _W	0.10 × V _W	3.75	8.4	N/D	6.5-fold	5.2-fold	1.4-fold
FB-1	11.0	1.0 × V _W	0.10 × V _W	3.51	13.9	N/D	3.1-fold	2.4-fold	2.4-fold
FB-2	20.5	1.0 × V _W	0.10 × V _W	3.64	8.6	N/D	3.4-fold	2.6-fold	2.6-fold
FB-3	20.6	1.0 × V _W	0.05 × V _W	3.76	5.4	N/D	4.6-fold	3.3-fold	3.3-fold
BR-B	4.5	1.0 × V _W	N/A	2.95	7.5	133	1.8 × 10 ¹³ (reference)	3.8 × 10 ¹² (reference)	3.8 × 10 ¹² (reference)
BR-P	25.8	10.8 × V _W	N/A	3.86	10.3	330	15.3-fold	13.2-fold	similar
BR-FB/P	16.5	5.0 × V _W	0.12 × V _W	3.65	8.8	305	8.3-fold	7.4-fold	1.5-fold

^a Normalized to a working volume of 35 mL for shake flask cultures and 1 L for bioreactor cultures, to account for differences in total virus production due to the variations in working volume.

^b Presented values correspond to fold-changes in comparison to low cell density batch references highlighted in gray (B-1 for shake flasks and BR-B for bioreactors).

VCD_{MAX}, maximum VCD; V_T, total volume of spent media (basal + feed); V_{feed}, total volume of concentrated feed added; HA_{MAX}, maximum HA titer, Y_T, total yield; CSVY, cell specific virus yield; CSVY_{IVP}, cell specific infectious virus yield; STY, space time yield; VP, volumetric productivity; V_W, working volume (final culture volume for fed-batch operations); N/A, not applicable; N/D, not determined.

5.6.4 IAV production in STR bioreactors using a TFDF system for perfusion and continuous harvesting of virus

Two IAV production runs were carried out using a 3 L benchtop bioreactor coupled to a TFDF system for cell retention and virus harvesting: a perfusion operation (BR-P), infected at 24×10^6 cells/mL; a hybrid fed-batch/perfusion operation (BR-FB/P) infected at 15×10^6 cells/mL. To serve as a reference for comparison, a batch bioreactor (BR-B), infected at 2×10^6 cells/mL with a dilution step at TOI, was carried out in a 1 L benchtop bioreactor.

Cells cultured in the batch bioreactor (BR-B) reached 4.5×10^6 cells/mL with an average t_D of 26 h and, after a MD step, were infected at around 2×10^6 cells/mL (Figure 5.5a). Glutamine and ammonium levels reached respectively lower and higher levels for the bioreactor when compared to the shake flask culture. This is mostly linked to the higher t_D and slightly higher cell densities reached in the bioreactor, as average cell specific consumption/production of those nutrients were overall lower for the bioreactor culture (Table B2). Nevertheless, ammonium levels remained below 4 mM in culture BR-B (Figure 5.5d). Although cell specific glucose consumption and lactate production were 2-fold higher for the bioreactor cultivation, accumulation of lactate remained below 30 mM (Figure B5). A maximum titer of $2.95 \log_{10}(\text{HAU}/100 \mu\text{L})$ was achieved at 44 hpi (Figure 5.5e).

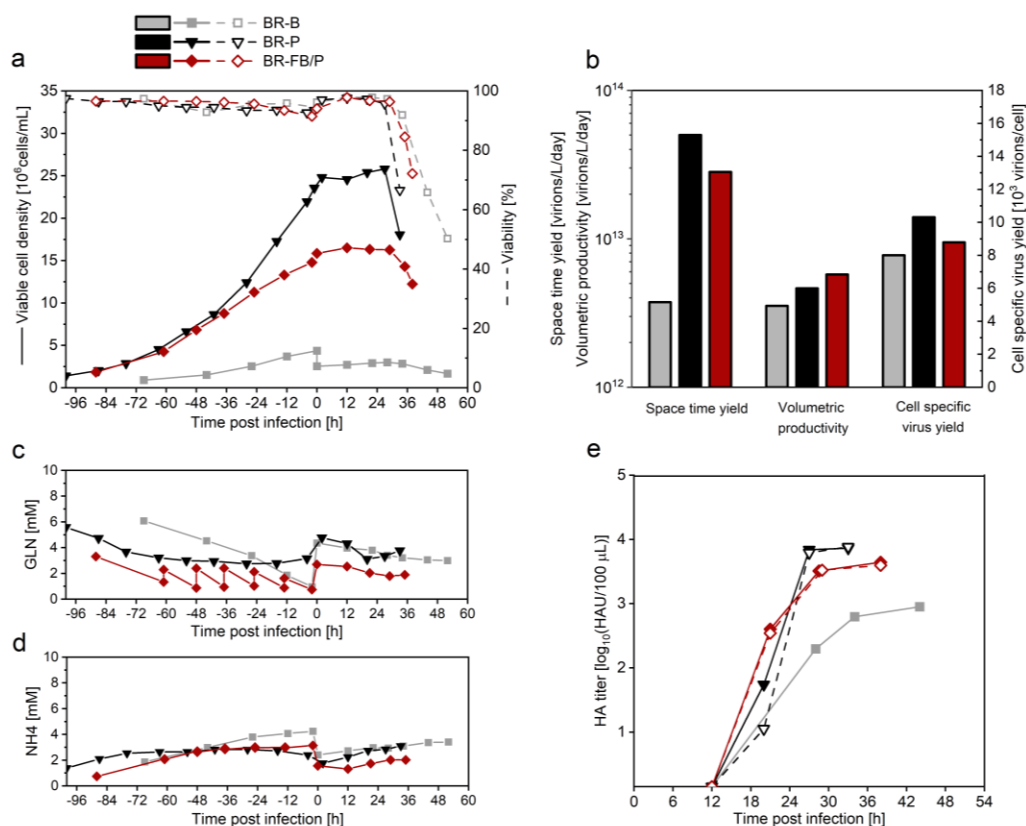


Figure 5.5 IAV production in MDCK.Xeno cells in benchtop bioreactors. In the batch operation (BR-B), cells were cultured in a 1 L STR bioreactor to 4.5×10^6 cells/mL and, after a MD step, infected at around 2×10^6 cells/mL. IAV production in perfusion mode (BR-P) was performed in a 3 L STR bioreactor, infected at 24×10^6 cells/mL. The perfusion was started 24 h after inoculation, and a CSPR of ~ 160 pL/cell/day was maintained through manual adjustment of the permeate flow rate. Medium exchange (1 RV) was performed at time of infection. IAV production in FB/P mode (BR-FB/P) was performed in a 3 L STR bioreactor, infected at 15×10^6 cells/mL. Concentrated feed was added to maintain glutamine concentration above 0.5 mM. Medium exchange (1 RV) was conducted at time of infection. Perfusion was started after infection (1.5–2.0 VVD) for continuous harvesting. Infections were performed with an MOI of 0.001 with addition of 20 U/mL of trypsin. At TOI, the temperature was reduced to 32°C. **a)** Viable cell density (full symbols) and viability (empty symbols), **b)** productivity, **c)** glutamine concentration, **d)** ammonium concentration, and **e)** HA titer in the bioreactor broth (full symbols) and permeate (empty symbols).

In the perfusion bioreactor (BR-P), cells grew exponentially with an average t_D of 24 h, reaching 24×10^6 cells/mL at TOI. A cell retention efficiency $> 99.9\%$ indicated that no loss of cells occurred due to membrane leakage, and transmembrane pressure (TMP) remained low throughout the run (< 0.01 psi during perfusion and ≤ 0.4 psi during ME and harvest steps). The manual step-wise control of the PR maintained an average CSPR of 163 ± 15 pL/cell/day during the growth phase

(Figure 5.6a), which was able to keep glutamine concentration above 2.5 mM and ammonium levels below 3 mM throughout the process (Figure 5.5c, Figure 5.5d). Before infection, a partial medium exchange of 1 RV was performed by increasing the permeate rate to 30 mL/min for approximately 40 min. At the TOI, the temperature was shifted to 32°C and the perfusion was turned off for 1 h to allow for virus entry into the cells. Cells continued to grow slowly after infection, reaching a maximum VCD of 25.8×10^6 cells/mL at 28 hpi (Figure 5.5a). A maximum titer of $3.86 \log_{10}(\text{HAU}/100 \mu\text{L})$ was reached at 33 hpi (Figure 5.5e). To evaluate virus retention by the TFDF membrane and its capacity for continuous harvesting, samples taken simultaneously from the bioreactor broth and the permeate line were measured. HA titer and infectious virus titers in the culture broth and permeate line were comparable or remained inside the assay error throughout the run (Figure 5.5e; Figure B7). Moreover, the HA titer in the bioreactor at TOH was comparable to the HA titer in the harvested material (Table B3). When compared to the batch bioreactor, the perfusion operation resulted in a 13-fold increase in STY, 15-fold increase in Y_T and, although it increased medium consumption by almost 11-fold, presented a comparable VP (Table 5.2, Figure 5.5b). Both CSVY and CSVY_{IVP} were comparable to the batch reference (Table 5.2).

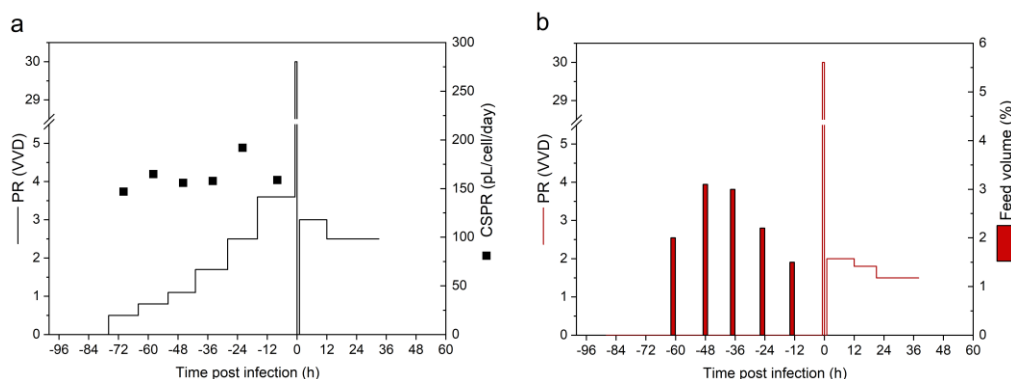


Figure 5.6 Feeding regimes for IAV production in 3 L STR bioreactors operated in perfusion (BR-P) and hybrid fed-batch/perfusion (BR-FB/P). For the BR-P operation, perfusion was started 24 h after inoculation, and a CSPP of ~160 pL/cell/day was maintained through manual adjustment of the permeate flow rate. For the BR-FB/P operation, concentrated feed was added during the growth phase to maintain glutamine concentration above 0.5 mM. Perfusion was started after infection for the continuous harvesting step. For both runs partial medium exchange (1 RV) was conducted at time of infection. **a)** Perfusion rate (PR) and cell specific perfusion rate (CSPP) for BR-P bioreactor and **b)** feed volume (as a percentage of the culture volume at time of feed) and PR for BR-FB/P bioreactor.

For BR-FB/P, cells grew exponentially with an average t_D of 24 h, reaching 15×10^6 cells/mL at TOI (Figure 5.5a). The feeding regime adopted during the growth phase (Figure 5.6b) maintained glutamine concentrations between 0.7–2.5 mM and ammonium levels lower than 3 mM (Figure 5.5c, Figure 5.5d). While q_{GLN} and q_{NH_4} were lower for the fed-batch bioreactor in comparison to the same strategy in shake flasks (FB/P-4), glucose consumption and lactate formation were higher for the bioreactor culture (Table B2). Still, glucose concentration remained above limiting values and, although lactate levels reached 30 mM at the TOI, partial medium exchange (permeate flow rate set to 30 mL/min for around 40 min) and the perfusion step after infection reduced it to less than 10 mM during the infection phase (Figure B5). As for BR-P, temperature was shifted to 32°C at TOI and the perfusion was turned off for 1 h (Figure 5.6b). A maximum VCD of 16.5×10^6 cells/mL was reached 12 hpi. Just as observed for the perfusion operation, no cell loss through the cell retention device was detected, and TMP values remained low throughout the process (< 0.01 psi during the infection phase, and ≤ 0.4 psi during ME and harvest steps). A maximum titer of $3.65 \log_{10}(\text{HAU}/100 \mu\text{L})$ was obtained. HA titer and infectious virus titers in the BR-FB/P culture broth and permeate line were comparable or remained inside the assay error throughout the run (Figure 5.5e; Figure B7). The same was true for the viral content before and after harvest (Table B3). In comparison to the batch bioreactor, the hybrid operation resulted in more than 8-fold increase in Y_T , 7.4-fold increase in STY and, although increasing medium consumption by almost 5-fold, a 1.5-fold increase in VP (Table 5.2). Both CSVY and CSVY_{IVP} values obtained for the hybrid bioreactor were comparable to the batch reference (Table 5.2).

5.7 Discussion

Although cell culture processes have been developed and licensed as a valuable alternative to traditional egg-based influenza vaccine production [8, 51], the development of simple, scalable, and cost-effective processes remains a challenge. Intensified processes must sustain high cell densities while avoiding the so-called “cell density effect”, characterized by a reduction in CSVY (virus production per cell), which has been related to nutrient limitation and accumulation of toxic metabolites in high cell density cultures [23, 134, 148]. The design of dynamic feeding strategies and the use of perfusion and fed-batch operations to support higher cell densities while limiting medium consumption and maximizing virus production is essential to enable the use of cell culture

processes in large-scale vaccine manufacturing. In this study, we have developed fed-batch-based strategies for IAV production and demonstrated their scalability in benchtop bioreactors. We have also evaluated the use of a TFDF system for continuous IAV harvesting in a perfusion process and a hybrid fed-batch process.

5.7.1 Development of a hybrid fed-batch/perfusion process for IAV production in shake flasks

One consideration for the implementation of perfusion processes is the higher cost and manufacturing logistics associated with medium consumption as well as handling and disposal of high volumes of fluid [21, 22]. In an attempt to decrease medium consumption while benefiting from the increase in productivity and lower accumulation of toxic by-products typically achieved with perfusion operations, several hybrid fed-batch/perfusion strategies have been reported. When applied to recombinant protein production, either a perfusion step or one or more medium exchange cycles performed during early-mid exponential growth phase resulted in an increase in volumetric and specific productivities when compared to traditional fed-batch – which was linked to the removal of toxic by-products and fresh nutrient supply – and lower medium consumption when compared to perfusion [42-44, 149]. Considering the short production phase characteristic of virus production processes, and to reduce the risk of degradation of the produced virus [25], we proposed a different approach. Therefore, a fed-batch operation was performed during the growth phase to increase the cell density while limiting medium consumption, and a medium exchange followed by perfusion was performed for the infection phase to alleviate by-product accumulation and decrease the time viruses are retained inside of the bioreactor. While a similar approach was conducted in a previous study with HEK293 cells [134], the faster growth and higher VCDs achieved with suspension MDCK cells and the resulting accumulation of toxic by-products require a more efficient dynamic feeding strategy for the fed-batch phase.

A low cell density batch culture and a semi-perfusion culture were performed in shake flasks, as previously described [34, 123], to serve as references for comparison of virus production. The obtained CSVY values (between $10.5\text{--}11.1 \times 10^3$ virions/cell) were comparable to what has been reported for IAV (H1N1) production in suspension MDCK cells [34, 38, 123]. Based on the glutamine profile obtained with the reference cultures, and to avoid nutrient limitations during the fed-batch phase, a concentrated feeding regime was established in initial FB/P cultures (FB/P-1

and FB/P-2) to maintain glutamine concentration above 2 mM. However, very high concentrations of ammonium were reached, of up-to 7.5 mM for condition FB/P-2 (Figure 5.2c), as well as high t_D (26 h and 30 h) when compared to the batch and semi-perfusion reference cultures (18 h and 21 h respectively for B-1 and P-1). In fact, studies have reported negative effects in cell growth and virus production for ammonium concentrations as low as 3 mM for different cell lines [135, 137]. As a ME step was performed at the TOI, followed by continuous harvesting of the produced virus, the CSVY obtained for the two hybrid conditions was comparable to the batch reference (Figure 5.2b, Table 5.2), indicating the absence of a cell density effect. However, as a complete medium exchange is hardly feasible in bioreactor cultures – instead, a partial medium exchange by the perfusion of 1 RV in a short interval is usually performed –, the high levels of ammonium attained in both shake-flask cultures could still affect virus production in a scale-up scenario.

In an attempt to limit ammonium formation by limiting glutamine supply [12, 135], addition of concentrated feed was performed in order to maintain lower levels of glutamine, above 0.5 mM for conditions FB/P-3 and FB/P-4, and above 0.2 mM for condition FB/P-5. Lower levels of ammonium were achieved during the growth phase (a maximum of 4.7 mM, Fig3.c), which reflected in t_D comparable to the semi-perfusion reference (22 h for conditions FB/P-3 and FB/P-4, and 21 h for condition FB/P-5). No meaningful difference was observed in the average $Y_{NH4/GLN}$ during the growth phase for cultures FB/P-1 and FB/P-2 (0.41 and 0.39) in comparison to cultures FB/P-3 and FB/P-4 (0.44 and 0.45), indicating that the lower glutamine concentrations in the medium did not alter the ratio of ammonium formed by glutamine consumption. However, while q_{GLN} was lower for cultures FB/P-3 and FB/P-4 (-0.48 and -0.45 pmol/cell/day compared to -0.58 and -0.54 pmol/cell/day for cultures FB/P-1 and FB/P-2), a higher q_{GLUC} and lower $Y_{LAC/GLUC}$ was observed, indicating a shift in metabolism towards a more efficient glucose utilization (Table B2). A similar trend was observed for a hybridoma cell line, with lower concentrations of glutamine in the culture medium leading to a shift to glucose consumption and lower $Y_{LAC/GLUC}$ values [12]. While the CSVYs of cultures FB/P-3 and FB/P-4 were comparable to the batch reference indicating no loss in specific productivity for the cultures infected at 15×10^6 cells/mL, a drop in CSVY was observed for culture FB/P-5, indicating the presence of a cell density effect when the infection was performed at 20×10^6 cells/mL. With the ME step performed and nutrients supplied through the continuous harvesting operation, neither a clear limitation of glutamine and glucose nor an accumulation of other measured metabolites (ammonium and lactate) was detected after infection.

Therefore, the drop in CSVY could be related to a shift in the metabolic state of cells caused by limitation of non-quantified nutrients and/or by-product formation before infection [134] as well as to accumulation of non-quantified inhibitors. Although it is unclear if using the same trypsin activity for low and high cell density cultures has an impact during the infection, the activity of trypsin used (20 U/mL) did not result in any limitations for the P-1 culture, infected at an even higher cell density. Therefore, the reduction in CSVY observed for culture FB/P-5 is most likely not related to trypsin limitations.

No difference in titer and productivity was observed when using perfusion medium supplemented with 3.5% concentrated feed for the perfusion step of the hybrid operation (FB/P-4) when compared to the non-supplemented medium (FB/P-3). However, as cells cultured in bioreactor often present slightly different metabolisms when compared to shake flask cultures [134], the use of supplemented medium was maintained when scaling up to benchtop bioreactor to insure the supply of non-quantified nutrients. The best performing hybrid feeding strategy, (FB/P-4), resulted in 7.4-fold increase in Y_T , almost 5-fold increase in STY and 1.5-fold increase in VP when compared to the batch reference. The dynamic feeding performed during the growth phase was able to sustain cell growth by supplying required nutrients without overfeeding, therefore limiting by-product accumulation. Similar dynamic feeding strategies have been shown to increase titer and productivity in antibody production processes with CHO cells [17].

5.7.2 Evaluation of fed-batch processes for IAV production in shake flasks

While the advantages of continuous processing have been reported in the production of both recombinant proteins and viruses [36, 61, 97, 150], the higher development complexity of these processes as well as the need for adapted facilities should not be underestimated [22, 133]. To provide alternatives for simpler and more robust operations, we evaluated if conventional and intensified fed-batch strategies would be capable of sustaining high cell densities while limiting by-product formation and maximizing virus production. Cells in condition FB-1 were infected at 5×10^6 cell/mL after a MD step. Although this strategy represented a less important increase in VCD when compared to the other proposed approaches, improvement in titers and productivity were achieved with a 3-fold and 2.4-fold increase in Y_T and VP, respectively, when compared to the low cell density batch reference (Figure 5.4b). Intensified fed-batch strategies, infected at 10×10^6 cells/mL after a MD step (FB-2) and 20×10^6 cells/mL (FB-3) were evaluated. Again, low values

of q_{GLN} (-0.29 and -0.27 pmol/cell/day for conditions FB-2 and FB-3, respectively) were followed by a more efficient consumption of glucose ($Y_{\text{LAC/GLUC}}$ of 0.16 and 0.17 for FB-2 and FB-3, respectively) (Table B2). Although no clear limitation in quantified nutrients (glutamine and glucose) nor accumulation of by-products (lactate and ammonium) was detected (Figure 5.4c; Figure 5.4d), a drop in CSVY was observed for both conditions clearly indicating some kind of cell density effect. This drop led to productivities comparable to condition FB-1 even though infections were performed at higher cell densities (Figure 5.4b). Moreover, a more pronounced decrease in CSVY was observed for condition FB-3 when compared to condition FB-2. In fact, CSVY values for both cultures suggest that the addition of fresh medium partially “restored” the cell specific productivity and, as the only difference between the latter was the dilution step at TOI, it is likely that this step either supplied a potential depleted nutrient or diluted potential toxic by-product(s). These results suggest that the development of concentrated feed medium specific for virus production processes could be a critical step towards the development of efficient fed-batch processes. Moreover, MDCK cells have been shown to secrete protease inhibitors that affect trypsin activity, therefore interfering with virus activation and propagation [151]. The more pronounced drop in CSVY observed for FB-3 could be related to a higher concentrations of protease inhibitors that were not diluted with the MD step. The effect of higher trypsin concentrations or multiple cycles of trypsin addition could be evaluated for high cell density cultures operated in fed-batch without medium exchange. As the fed-batch processes screened in shake flasks did not meaningfully improve virus production, this mode of operation was not further explored in a bioreactor culture.

5.7.3 IAV production in STR bioreactors using a TFDF system for perfusion and continuous harvesting of virus

Interest over integrated processing of viral particles has increased over the last years, as this type of process favors product quality by reducing processing and hold times [35, 36, 150]. During upstream processing, the choice of a cell retention device that allows for continuous harvesting of the produced material is critical to allow integration to latter downstream steps. While membrane-based cell retention devices, such as the alternating tangential flow filtration (ATF), are usually preferred due to better scalability, IAV retention was reported for pore sizes $< 0.5 \mu\text{m}$ [27, 38]. In this study, we evaluated the use of a TFDF system as cell retention device for continuous harvesting

and clarification of IAV in two intensified processes: a perfusion process infected at 24×10^6 cells/mL (BR-P) and a hybrid process operated in FB/P, infected at 15×10^6 cells/mL (BR-FB/P). A low cell density batch bioreactor culture was performed as a reference for comparison.

The reference bioreactor batch culture presented H1N1 virus titers comparable to other batch processes performed with suspension MDCK cells derived from the ECACC adherent cell line, which range from 2.74–3.34 $\log_{10}(\text{HAU}/100 \text{ } \mu\text{L})$ [34, 142, 143]. Suspension MDCK cells cultivated in perfusion using the TFDF system reached high cell densities with similar growth behavior of MDCK cells cultivated using an ATF system [34, 115]. The intensified perfusion strategy was able to support IAV production for infections performed at 24×10^6 cells/mL and successfully overcome the cell density effect, resulting in more than 15-fold increase in Y_T and 13-fold increase in STY when compared to the batch reference (Table 5.2). Similar perfusion processes employing different membrane-based cell retention devices for the production of IAV and IAV defective interfering particles (DIPs) in suspension MDCK cells were able to overcome the cell density effect resulting in significant increase in productivity [34, 38, 115]. However, total or partial virus retention due to progressive membrane clogging was reported for almost all of the membranes tested. Continuous harvesting of IAV DIPs produced in membrane-based perfusion cultures of MDCK-PB2 cells was possible when using an ATF system equipped with a VHU membrane ($\sim 10 \text{ } \mu\text{m}$ pore size) [115]. However, the use of membranes with larger pore sizes increases the risk of cell leakage and particle breakthrough, notably at the higher PRs used for the ME step [115].

The intensified BR-FB/P operation supported successful IAV production in a bioreactor culture infected at 15×10^6 cells/mL, overcoming the cell density effect, and resulting in more than 8-fold increase in Y_T and 7.4-fold increase in STY. Although the perfusion operation resulted in a 75% higher STY when compared to the hybrid operation, medium consumption was more than doubled ($10.8 \times V_W$ for the BR-P compared to $5.0 \times V_W$ for BR-FB/P), which resulted in a higher VP for the hybrid operation (Table 5.2). While dynamic feeding strategies based on growth rate and cell specific consumption/production rates are critical to limit by-product accumulation and avoid overfeeding, they might increase the complexity of the operation by requiring frequent and online assessment of nutrient concentration. Moreover, as growth and consumption rates vary significantly in fed-batch processes depending on the current stage of the culture (early, mid or late-exponential), an efficient feeding strategy must adapt to the current culture requirements. A

cell specific feeding rate (CSFR; pL/cell/day) defined for a specific process and varying for different VCD ranges could then be used to simplify the feeding process. Based on the feeding volume added for the same FB/P strategy applied in shake flask and bioreactor (FB/P-3, FB/P-4 and BR-FB/P) the corresponding CSFR for different VCD ranges was calculated (Table B4). Although cells cultured in different scales presented variations in glucose and lactate metabolisms, the results show that the calculated CSFR for each VCD range was comparable between the different cultures, suggesting that a CSFR table could be used to simplify the dynamic feeding strategy developed. The use of a capacitance probe to control feed addition based on cell growth could further improve the feeding process [38, 115]. While cell specific production of lactate was higher for all bioreactor cultures when compared to shake flask cultivations (Table B2), lactate concentrations remained below levels that could be harmful for virus production [135] (below 20 mM) during the infection phase for all three bioreactor runs.

While each process was performed as a single replicate both in shake flask and bioreactor, comparable results for growth and productivity were obtained for the same feeding strategy in different scales. Moreover, the extent of the improvement in production (more than 15-fold and 8-fold increase in total yield for perfusion and hybrid operations, respectively) strongly attests for the performance of the developed strategies in comparison to the batch reference. The CSVY obtained for the bioreactor runs was comparable to what has been reported for the same cell line (MDCK.Xeno) producing IAV in semi-perfusion cultures [123], as well as to a different suspension MDCK cell line (derived from adherent MDCK cells from the American-Type Culture Collection (ATCC)) producing IAV in perfusion cultivations [38]. No loss of cells through the cell retention device was detected for both bioreactor runs, and TMP values remained low throughout the process (growth and production phases as well as final harvest step). Virus titer in the bioreactor broth and permeate line, measured through the HA titer and the infectious titer, were comparable throughout the production phase for both BR-P and BR-FB/P runs, demonstrating that no virus loss nor retention occurred when employing the TFDF membrane for clarification and continuous harvesting of IAV, even in cultures reaching almost 26×10^6 cells/mL. While the same was reported by Silva, et al. [134] when employing the TFDF system for IAV production in suspension HEK293 cells infected at 10×10^6 cells/mL, our results demonstrate that TFDF membranes are suitable for continuous harvest and clarification of IAVs at even higher cell densities. High cell

density perfusion operations with continuous harvesting of LVs [32, 117], AAVs [33], and VSV [126] using TFDF membranes are reported.

5.8 Conclusion

In this study, we have demonstrated a scalable hybrid fed-batch/perfusion process capable of sustaining intensified IAV production in suspension MDCK cell cultures infected at 15×10^6 cells/mL. The developed feeding regime contributed to limit the accumulation of toxic by-products, notably ammonium, and overcome the cell density effect while reducing total medium consumption. The hybrid operation resulted in more than 8-fold increase in Y_T when compared to a batch reference. We have also reported a perfusion process able to support IAV production in cultures infected at 24×10^6 cells/mL with no loss in cell specific productivity, resulting in more than 15-fold increase in Y_T when compared to a batch reference. Our results suggest that, while perfusion operations are better suited to maximize the production for a given footprint, hybrid processes are a valuable option when medium consumption and handling are a concern. When evaluating IAV production in high cell density cultures operated in fed-batch, virus production was impacted by the cell density effect. As the observed concentrations for quantified nutrients and metabolites did not indicate limitation nor inhibition, our results suggest that other non-quantified nutrients and metabolites were responsible for the observed effect. The development of concentrated feed medium specifically tailored for virus manufacturing as well as more studies on the design of dynamic feeding strategies could help to support virus production in fed-batch processes at higher cell densities. Finally, our results support that TFDF membranes are suitable for continuous harvesting of IAVs in high cell density processes, with no virus retention nor loss during production phase and final harvest step. Overall, the use of a TFDF system as a cell retention device to allow for an integrated production of influenza vaccines could lead the way in manufacturing of next generation IAV vaccines.

5.9 Supplementary information

Supplementary material is presented in appendix B.

5.10 Acknowledgements

The authors would like to acknowledge Repligen Corp for providing the KrosFlo TFDF lab unit and custom TFDF membrane modules and Xell AG (Sartorius) for providing the concentrated feed

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5.11 Author contributions

Cristina A. T. Silva: Conceptualization, Methodology, Investigation, Validation, Writing–Original Draft, Writing–Review & Editing, Project administration **Lars Pelz:** Methodology, Investigation, Writing–Review & Editing **Tilia Zinnecker:** Investigation, Writing–Review & Editing **Sven Göbel:** Methodology, Writing–Review & Editing **Ivonne Genzel:** Writing–Review & Editing, Supervision **Udo Reichl:** Writing–Review & Editing, Supervision **Béla Brühlmann:** Resources, Writing–Review & Editing **Rachel Legmann:** Resources, Writing–Review & Editing, **Amine A. Kamen:** Writing–Review & Editing, Supervision, **Olivier Henry:** Conceptualization, Writing–Review & Editing, Supervision, Project administration. All authors read and approved the manuscript.

5.12 Conflict of interest

B. Brühlmann and R. Legmann are employees of Repligen, where the Krosflo TFDF system has been developed.

5.13 Key points

In this article, we have evaluated fed-batch-based feeding strategies for intensified IAV production in suspension MDCK cells. We have also evaluated the use of a TFDF module as cell retention device in a perfusion and a hybrid fed-batch/perfusion process performed using a bench-top bioreactor.

A dynamic feeding strategy, controlled based on glutamine consumption, was applied in processes operated in hybrid fed-batch/perfusion and fed-batch mode.

- When applied to cultures operated in hybrid fed-batch/perfusion mode, the dynamic feeding strategy was able to sustain cell growth by limiting by-product formation and insuring the supply of nutrients during the growth phase. After infection, a shift to perfusion mode reduced the concentration of toxic by-products and allowed for continuous harvest of the produced virus. Operation in hybrid mode was able to overcome the cell density effect in cultures infected at up-to 15×10^6 cells/mL performed both in shake flask and bioreactor. Up-to 8-fold increase in total production was reported for the hybrid process when compared to a low cell density batch reference.

- When applied for IAV production in HCD cultures operated in fed-batch, virus production was significantly impacted by the cell density effect. While the dynamic feeding strategy was able to sustain cell growth and limit ammonium and lactate accumulation, it was not able to sustain effective virus production. The results suggested that non-quantified nutrients and/or toxic metabolites could be responsible for the observed effect.

A TFDF module was evaluated as cell retention device for IAV production in HCD cultures of MDCK cells.

- When applied as cell retention device and harvest/clarification unit to a perfusion process, no loss nor retention of viruses was observed for a culture infected at 24×10^6 cells/mL. The perfusion operation resulted in more than 15-fold increase in total production when compared to a low cell density batch reference.
- When applied as cell retention device and harvest/clarification unit to a hybrid fed-batch/perfusion process, no loss nor retention of viruses was observed for a culture infected at 15×10^6 cells/mL. The hybrid operation resulted in more than 8-fold increase in total production when compared to a low cell density batch reference.

Our results suggested that while perfusion operations are preferable to maximize virus production for a given footprint, hybrid operations are a valuable alternative when medium consumption and handling must be minimized.

CHAPTER 6 ARTICLE 4 : FED-BATCH STRATEGIES FOR INTENSIFIED rVSV VECTOR PRODUCTION IN HIGH CELL DENSITY CULTURES OF SUSPENSION HEK293 CELLS

6.1 Article presentation

The article presented in this chapter was submitted for publication to the journal *Biotechnology Progress* on June 3, 2024. Here, we apply and evaluate the fed-batch-based strategies developed in the previous chapters to intensify the production of a recombinant viral vector based on the vesicular stomatitis virus (rVSV) using suspension HEK293 cells as substrate. The main goal of this study was to evaluate if the fed-batch-based strategies developed for influenza virus production were applicable to intensify the production of other viruses of interest.

In the previous chapters, we have developed and described dynamic feeding strategies in which feed addition was performed based on the growth rate and specific consumption rates for main metabolites. For that, the concentration of key nutrients and cells were assessed during the process, at every sample point, and used to calculate the amount of feed to be added at a given interval. In order to simplify the feeding strategy, and based on the accumulated data for growth, nutrient consumption and metabolite formation generated on chapter 4 for HEK293 cells, we have defined a range of values for an average cell specific feeding rate (CSFR; pL/cell/day), which allows feeding to be performed exclusively based on cell growth.

In this study, we have evaluated a CSFR-based fed-batch strategy for the production of rVSV vectors in high cell density cultures of HEK293 cells. We have employed Ambr250 modular bioreactors to evaluate cultures operated in fed-batch and hybrid fed-batch perfusion infected at two different cell densities and using two different CSFR values.

6.2 Fed-Batch Strategies for Intensified rVSV Vector Production in High Cell Density Cultures of Suspension HEK293 Cells

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6.3 Abstract

Vesicular stomatitis virus (VSV) has been increasingly demonstrated as a promising viral vector platform. As the interest over this modality for vaccine and gene therapy applications increases, the need for intensified processes to produce these vectors emerge. In this study, we develop fed-batch-based operations to intensify the production of a recombinant VSV-based vaccine candidate (rVSV-SARS-CoV-2) in suspension cultures of HEK293 cells. A feeding strategy, in which a commercial concentrated medium was added to cultures based on cell growth through a fixed cell specific feeding rate (CSFR), was applied for the development of two different processes using Ambr250 modular bioreactors. Cultures operated in hybrid fed-batch/perfusion (FB/P) or fed-batch (FB) were able to sustain infections performed at 8.0×10^6 cells/mL, respectively resulting in 3.9-fold and 5.0-fold increase in total yield (Y_T) and 1.7 and 5.6-fold increase in volumetric productivity (VP) when compared to a batch reference. A maximum viral titer of 4.5×10^{10} TCID₅₀/mL was reached, which is comparable or higher than other processes for VSV production in different cell lines. Overall, our study reports efficient fed-batch options to intensify the production of a rVSV-based vaccine candidate in suspension HEK293 cells.

Keywords: process intensification, fed-batch, viral vector, suspension cells, high cell density, HEK293 cells

6.4 Introduction

The interest for viral vectors for vaccine applications have greatly increased over the last decades. The approval and broad use of two viral vectored vaccines to fight the recent COVID-19 pandemic – ChAdOx1 (Oxford-AstraZeneca) and Jcovden (Janssen), both based on adenovirus technology – highlights the safety and responsiveness of this modality [152, 153]. Vesicular stomatitis virus (VSV) is a promising vaccine platform, notably due to the low prevalence of pre-existing immunity in humans and its ability to replicate to high titers in mammalian cell culture [31, 87, 154-156]. The approval of the VSV-based Ebola vaccine rVSV-ZEBOV (ERVEBO®) in 2019 both by the Food and Drug Administration and the European Medicines Agency underlines the potential of

this production platform [157-159]. Besides its application in vaccine manufacturing, the use of VSV in gene therapy or as an oncolytic virus has also shown promising results [160, 161].

Large scale production of rVSV-ZEBOV is conventionally performed using adherent Vero cells, currently the most accepted continuous cell line for vaccine production [162]. However, as the use of cells growing in suspension is more advantageous regarding process scale-up, the screening and development of suspension cell lines is critical. Production of recombinant VSV vectors in different suspension cell lines, including Vero cells, BHK-21, AGE1.CR and HEK293 cells, is reported [31, 126, 155, 163-165]. Of interest, comparable or even higher titers were reported for VSV production in Vero cells adapted to suspension growth, when compared to adherent Vero cells [87, 163, 166]. However, the high doubling time reported for the suspension-adapted cells (40–65 hours, depending on the culture medium employed) limits the cell densities that can be achieved, underscoring the need for further cell line and medium development. Suspension HEK293 cells, derived from human embryonic kidney cells, are a broadly accepted expression platform for the production of recombinant proteins and viral vectors, being able to reach high cell densities in serum-free medium [105-108]. Production of VSV in suspension HEK293 cells reaching high virus titers ($0.1\text{--}1.0 \times 10^9$ TCID₅₀/mL for low cell density batch cultures, depending on the vector construct) has been reported [31, 126, 155, 164], highlighting the potential of this cell substrate.

To keep up with the demand, cell culture processes for viral vector production must be intensified. This is typically achieved by increasing the cell concentration at infection while avoiding, or at least minimizing, the so-called “cell density effect”, characterized by a drop in the cell specific virus yield (CSVY; virus/cell) in cultures infected at higher cell densities [23, 134, 148, 167]. To sustain higher cell concentrations, fed-batch or perfusion operations can be used. While perfusion operations typically allow for higher cell densities and productivities with small footprints, the high volumes of medium consumed increase the final costs and the complexity of the process [18, 20, 22]. Fed-batch operations, however, balance simpler processes and reduced medium consumption with more limited cell growth and production [16, 17, 21, 22, 39]. Hybrid processes, which combine both strategies, have the potential to sustain higher cell densities and productivities while limiting medium consumption [42, 43, 134].

In this study we develop efficient fed-batch-based processes to intensify the production of a rVSV-based vaccine candidate, rVSV-SARS-CoV-2, in HEK293SF cells using Ambr250 modular

bioreactors. To simplify the feeding process while avoiding overfeeding, addition of concentrated feed was performed based on cell growth through a cell specific feeding rate (CSFR; pL/cell/day), defined based on the specific nutrient requirements of the cell line.

6.5 Materials and Methods

6.5.1 Cell line and virus

HEK293 clone 293SF-3F6 [168], here referred as HEK293SF cells, were kindly provided by the National Research Council Canada and were cultivated in serum-free medium HyClone HyCell TransFx-H (Cytiva Life Sciences, USA) supplemented with 4 mM L-Glutamine (Gibco, USA) and 0.1% Kolliphor 188 (Sigma-Aldrich, USA). Cells were maintained in 125 mL shake flasks (25 mL working volume) in an orbital shaker incubator at 135 rpm, 37°C, and 5% CO₂. Cell counting was performed using a Vi-Cell XR Cell Viability analyzer (Beckman Coulter, USA) and main nutrients and metabolites (glutamine, ammonium, glucose and lactate) were quantified using a blood analyzer BioProfile® FLEX2 (Nova Biomedical, USA). Average specific consumption/production rates for main metabolites were calculated as previously described by Silva, et al. [134].

The temperature restricted rVSV-vectored COVID-19 vaccine candidate rVSVInd-msp-SF-Gtc (hereafter referred as rVSV-SARS-CoV-2), described in detail by Kiesslich, et al. [163], was derived from a virus stock produced in BHK-21 cells and adapted to HEK293SF cells by two consecutive passages. Due to the temperature sensitivity of this construct, the temperature was shifted to 31°C after infection for all the strategies tested. Infections were performed with a multiplicity of infection (MOI; virus/cell) varying between 0.01–0.001. Samples for virus quantification were collected, centrifuged (2000 × g, 5 min), aliquoted, and stored at -80°C until further analysis.

6.5.2 rVSV-SARS-CoV-2 production in shake flasks

Production of rVSV in shake flasks was performed in 125 mL shake flasks (25 mL working volume) using the conditions described above. To evaluate the effect of different MOIs, cultures operated in batch were infected at low cell densities, between $1.0\text{--}1.5 \times 10^6$ cells/mL, with MOIs of 0.001 or 0.01. After infection, the temperature was shifted to 31°C.

6.5.3 rVSV-SARS-CoV-2 production in Ambr250 Modular Bioreactors

Fed-batch-based feeding strategies were evaluated using an Ambr250 modular bioreactor system (Sartorius Stedim, Germany). Single use 350 mL baffled vessels (250 mL working volume) equipped with two pitched-blade impellers (26 mm diameter) were used. The bioreactors were maintained at 37°C, pH 7.1, and stirring speed of 190 rpm. After infection, the temperature was controlled at 31°C. The DO was maintained at 50% air saturation by sparging air and/or pure oxygen in a cascade control, and the pH was controlled with the addition of NaHCO₃ (70g/L) or CO₂ added to the headspace.

Ambr250 cultures were operated in batch (B), fed-batch (FB) and hybrid fed-batch/perfusion (FB/P). A detailed description of the feeding strategies tested is presented in Table 6.1. The bioreactor operated in batch was seeded at 0.3×10^6 cells/mL in fresh medium and, at around 1.5×10^6 cells/mL, infected with an MOI of 0.001 without medium exchange.

Fed-batch cultures were started at 2.3×10^6 cells/mL in fresh medium. During the growth phase, the same feeding strategy was applied for cultures operated in FB/P and FB: addition of commercial concentrated serum-free feed HyClone Cell Boost™ 5 (Cytiva Life Sciences, USA) supplemented with 30 mM L-Glutamine (Gibco, USA) was performed every 12 hours, based on a pre-defined cell specific feeding rate (CSFR; pL/cell/day), as presented in Table 6.1. The CSFR values adopted were defined based on preliminary experiments: briefly, HEK293SF cells were cultured in fed-batch mode and concentrated feed addition was performed based on the growth rate and the cell specific glutamine consumption rate while assuming that other nutrients were consumed at a similar rate, as described elsewhere [134]. Based on the volumes of feed added, an average CSFR value was calculated and applied in the present study. The volume of concentrated feed to be added at a given time (V_F) was calculated as follows:

$$V_F = X_{ave} * V_R * CSFR * \Delta t \quad (6.1)$$

$$X_{ave} = \frac{X_n}{2} * (e^{\mu * \Delta t} + 1) \quad (6.2)$$

where X_{ave} is the average cell concentration predicted for the given interval, V_R is the current volume in the reactor, Δt is the interval of time until the next feed, and μ is the cell specific growth rate calculated for the previous interval.

Following infection, different feeding regimes were adopted for FB/P and FB cultures. After infection of cultures operated in FB/P, performed between $8.0\text{--}10.0 \times 10^6$ cells/mL with an MOI of 0.001, a semi-perfusion phase was started. Cultures were semi-continuously harvested with a perfusion rate (PR) of 0.5 vessel volumes per day (VVD). For that, a determined volume of the culture was collected in spin flasks, centrifuged ($300 \times g$, 3 min), and the supernatant was collected and sampled (permeate pool). Pelleted cells were resuspended in a corresponding volume of fresh medium and returned to the culture vessel. On the other hand, FB cultures continued to be operated in fed-batch after infection, performed at around 8.0×10^6 cells/mL. Due to the reduction in growth and metabolism caused by the temperature shift, addition of concentrated feed after infection was performed based on a fixed percentage. A volume of concentrated feed corresponding to 5% of the culture volume was added both at the time of infection and at 24 hours post infection (hpi). An MOI of 0.01 was chosen for cultures operated in FB to limit the accumulation of toxic by-products after infection by shortening the production phase. The total volume of concentrated feed added in the different cultures varied slightly depending on the condition tested, corresponding to approximately 25% of the final volume.

6.5.4 Virus quantification

Infectious virus particles were quantified by TCID₅₀. Briefly, adherent HEK293 cells were cultured in DMEM medium (Gibco, USA) supplemented with 4 mM GlutaMAX (Gibco, USA) and 1% Pen/Strep (Gibco, USA). For the assay, 100 μL /well of cell suspension were seeded in a 96-well plate, with a seeding density of approximately 2.0×10^4 cells/well. After 24 hours, wells were infected with 100 μL of 10-fold serial dilutions of infectious culture supernatant, with 8 replicates per dilution. Plates were incubated at 31°C for up to 7 days and evaluated under standard light microscope on days 4 and 7 for the presence/absence of cytopathic effect in each well. TCID₅₀ titers were calculated following the Reed-Muench method.

6.5.5 Productivity evaluation

The total yield of virus (Y_T) was calculated based on the TCID₅₀ titers, as follows:

$$Y_T = C_R * V_R + \sum C_P * V_P \quad (6.3)$$

where C_R is the concentration of virus in the reactor at a given time, C_P is the concentration of virus in the permeate pool for a given time interval, V_R is the current culture volume, V_P is the permeate pool volume.

The productivity of the different processes was assessed through the cell specific virus yield (CSVY; TCID₅₀/cell), the volumetric productivity (VP; TCID₅₀/L/day) and the space time yield (STY; TCID₅₀/L/day), calculated as follows:

$$CSVY = \frac{Y_T}{X_T * V_W} \quad (6.4)$$

$$VP = \frac{Y_T}{V_T * t_T} \quad (6.5)$$

$$STY = \frac{Y_T}{V_W * t_T} \quad (6.6)$$

where X_T is the maximum total cell density, V_W is the working volume, V_T is the total volume of medium consumed, and t_T is the total duration of the culture.

6.6 Results

6.6.1 rVSV-SARS-CoV-2 production in shake flasks

To rapidly assess the effect of MOI on rVSV-SARS-CoV-2 production in HEK293SF cells, shake flask cultures operated in batch were infected at low cell densities, varying between $1.0\text{--}1.5 \times 10^6$ cells/mL, with an MOI of 0.01 or 0.001 (Figure 6.1). Peak virus production was achieved faster for cultures infected with a higher MOI, after 31–36 hpi for an MOI of 0.01 compared to 48–56 hpi for an MOI of 0.001 (Figure 6.1). However, as the infection with different MOIs did not result in meaningful differences in titer, the lower value (0.001) was chosen for the following experiments since lower MOIs are typically preferred to reduce the required volume of viral seed stock.

Maximum titers of 5.5×10^9 TCID₅₀/mL and 1.1×10^{10} TCID₅₀/mL were respectively obtained for shake flask cultures infected at 1.0×10^6 and 1.5×10^6 cells/mL.

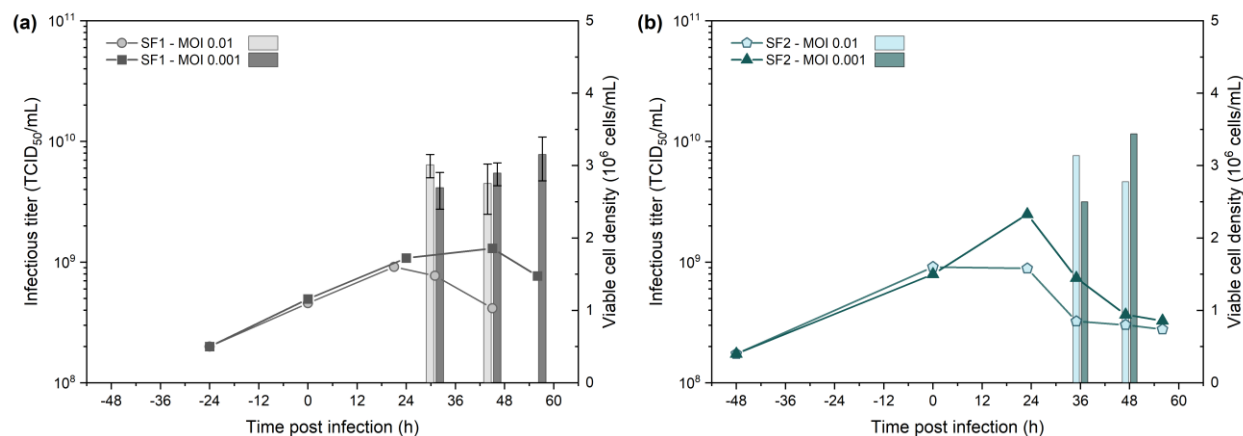


Figure 6.1 Production of rVSV-SARS-CoV-2 in suspension HEK293SF cell cultures performed in 125 mL shake flasks with different MOIs (0.001 and 0.01). Viable cell density (symbols) and virus titer (bars) for batch cultures infected at **(a)** 1.0×10^6 cells/mL (SF1; mean and standard deviation for two independent samples) and **(b)** 1.5×10^6 cells/mL (SF2; single replicate).

6.6.2 Evaluation of fed-batch-based strategies for rVSV-SARS-CoV-2 production in Ambr250 modular bioreactors

A conventional low cell density batch process was performed as a reference using the Ambr250 modular bioreactor system. Cells were seeded at 0.3×10^6 cells/mL and, after growing exponentially to 1.5×10^6 cells/mL, were infected with an MOI of 0.001 (Figure 6.2a). Glutamine and glucose concentrations remained above limiting values throughout the culture indicating the absence of main nutrient limitation. Ammonium and lactate concentrations remained below levels that could be harmful for virus production [135], respectively below 3 mM and 20 mM (Figure 6.4a, Figure 6.4b). A maximum virus titer of 9.0×10^9 TCID₅₀/mL was achieved at 56 hpi (Figure 6.2b) corresponding to a CSVY of 3896 TCID₅₀/cell (Table 6.1).

Table 6.1 Summary of culture conditions and results for rVSV-SARS-CoV-2 production in Ambr250 modular bioreactors.

Code	B	FB/P-1	FB/P-2	FB-1	FB-2
Growth phase	Batch	Fed-batch (20 pL/cell/day)	Fed-batch (20 pL/cell/day)	Fed-batch (15 pL/cell/day)	Fed-batch (20 pL/cell/day)
Infection phase	Batch	Semi-perfusion (PR 0.5 VVD)	Semi-perfusion (PR 0.5 VVD)	Fed-batch	Fed-batch
VCD ₀ (10 ⁶ cells/mL)	0.3	2.3	2.3	2.3	2.3
VCD _{TOI} (10 ⁶ cells/mL)	1.5	10.0	8.0	8.0	8.0
CSVY (TCID ₅₀ /cell)	3896	2353	2718	4773	4074
Y _T (TCID ₅₀) ^a	2.2 × 10 ¹² (reference)	3.8-fold	3.9-fold	5.0-fold	4.4-fold
VP (TCID ₅₀ /L/day) ^a	1.9 × 10 ¹² (reference)	1.6-fold	1.7-fold	5.6-fold	4.7-fold
STY (TCID ₅₀ /L/day) ^a	1.9 × 10 ¹² (reference)	3.4-fold	3.5-fold	5.6-fold	4.7-fold
Time of harvest	56 hpi	64 hpi	64 hpi	48 hpi	48 hpi
Total volume of medium ^b	1 × V _W	2 × V _W	2 × V _W	1 × V _W	1 × V _W

^a From the second column onwards, presented values correspond to fold-changes in comparison to the low cell density batch reference (B) highlighted in gray.

^b Basal + feed medium.

VCD₀, VCD at inoculation; VCD_{TOI}, VCD at time of infection; CSVY, cell specific virus yield; Y_T, total yield; VP, volumetric productivity; STY, space time yield; PR, perfusion rate; VVD, vessel volumes per day; V_W, working volume; CSFR, cell specific feeding rate; B, batch; FB, fed-batch; FB/P, hybrid fed-batch/perfusion.

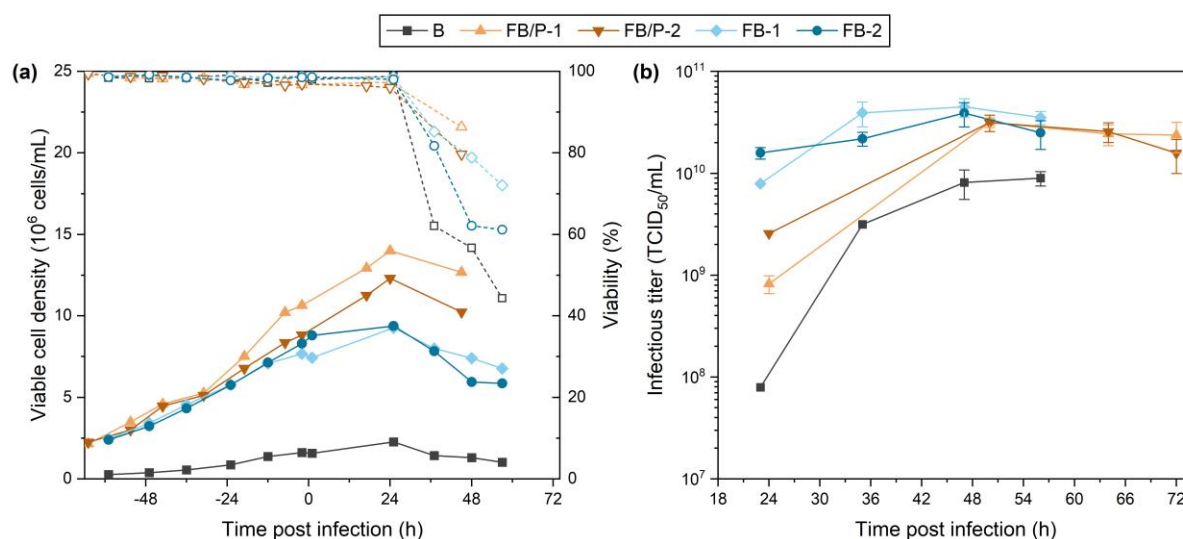


Figure 6.2 rVSV-SARS-CoV-2 production in HEK293SF cells performed in Ambr250 modular bioreactor system, operated in batch (B, infected at 1.5×10^6 cells/mL with an MOI of 0.001), hybrid fed-batch/perfusion (FB/P-1 and FB/P-2, infected between $8.0\text{--}10.0 \times 10^6$ cells/mL with an MOI of 0.001), and fed-batch (FB-1 and FB-2, infected at around 8.0×10^6 cells/mL with an MOI

of 0.01). **(a)** Viable cell density (full) and viability (empty) and **(b)** rVSV-SARS-CoV-2 titer (mean and standard deviation for two independent samples).

FB/P cultures were inoculated at 2.3×10^6 cells/mL. During the growth phase, a volume of concentrated commercial feed corresponding to a CSFR of 20 pL/cell/day was added every 12 hours. After infection, performed either at 10.0×10^6 cells/mL (FB/P-1) or 8.0×10^6 cells/mL (FB/P-2) with an MOI of 0.001, a semi-perfusion operation with a PR of 0.5 VVD was initiated for continuous harvest of the produced virus. Cells reached maximal densities of 14.0×10^6 cell/mL and 12.3×10^6 cells/mL at 24 hpi, respectively for conditions FB/P-1 and FB/P-2 (Figure 6.2a). Concentrations of glutamine and glucose did not suggest nutrient limitations during the cultivation for both conditions (Figure 6.4c, Figure 6.4d). While maximum virus titers of 3.1×10^{10} TCID₅₀/mL were achieved at 50 hpi for both conditions (Figure 6.2b), maximum production was only attained at 64 hpi due to the inherent dilution effect related to the semi-perfusion operation. A CSVY of 2353 TCID₅₀/cell and 2718 TCID₅₀/cell was obtained for FB/P-1 and FB/P-2 respectively, corresponding to a decrease of up to 40% when compared to the batch reference (Table 6.1). This drop in CSVY indicates that some kind of cell density effect occurred for both cultures operated in FB/P and this effect seemed to be more pronounced for cells infected at 10.0×10^6 cells/mL (FB/P-1). While ammonium levels remained below 3 mM (Figure 6.4c), lactate concentrations of almost 40 mM were reached for condition FB/P-1 and of 25 mM for FB/P-2 (Figure 6.4d), which could explain, at least in part, the more pronounced decrease in specific production for the first. Nevertheless, the best performing hybrid culture (FB/P-2, infected at 8.0×10^6 cells/mL) resulted in almost 4-fold increase in Y_T (Table 6.1), 3.5-fold increase in STY and 1.7-fold increase in VP when compared to the batch reference (Figure 6.3).

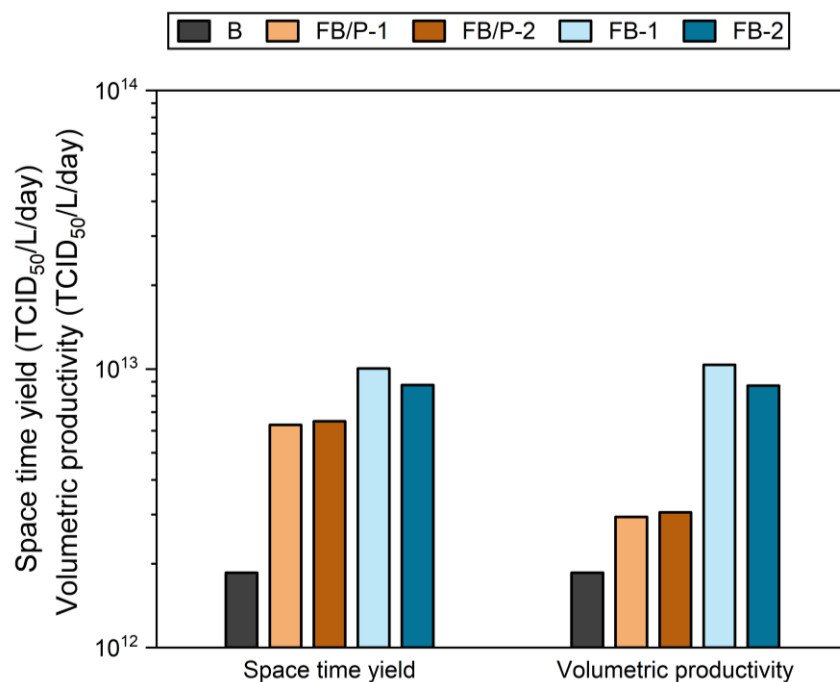


Figure 6.3 Space time yield (STY) and volumetric productivity (VP) of HEK293SF cultures producing rVSV-SARS-CoV-2 operated in batch (B, infected at 1.5×10^6 cells/mL with an MOI of 0.001), hybrid fed-batch/perfusion (FB/P-1 and FB/P-2, infected between $8.0\text{--}10.0 \times 10^6$ cells/mL with an MOI of 0.001), and fed-batch (FB-1 and FB-2, infected at 8.0×10^6 cells/mL with an MOI of 0.01).

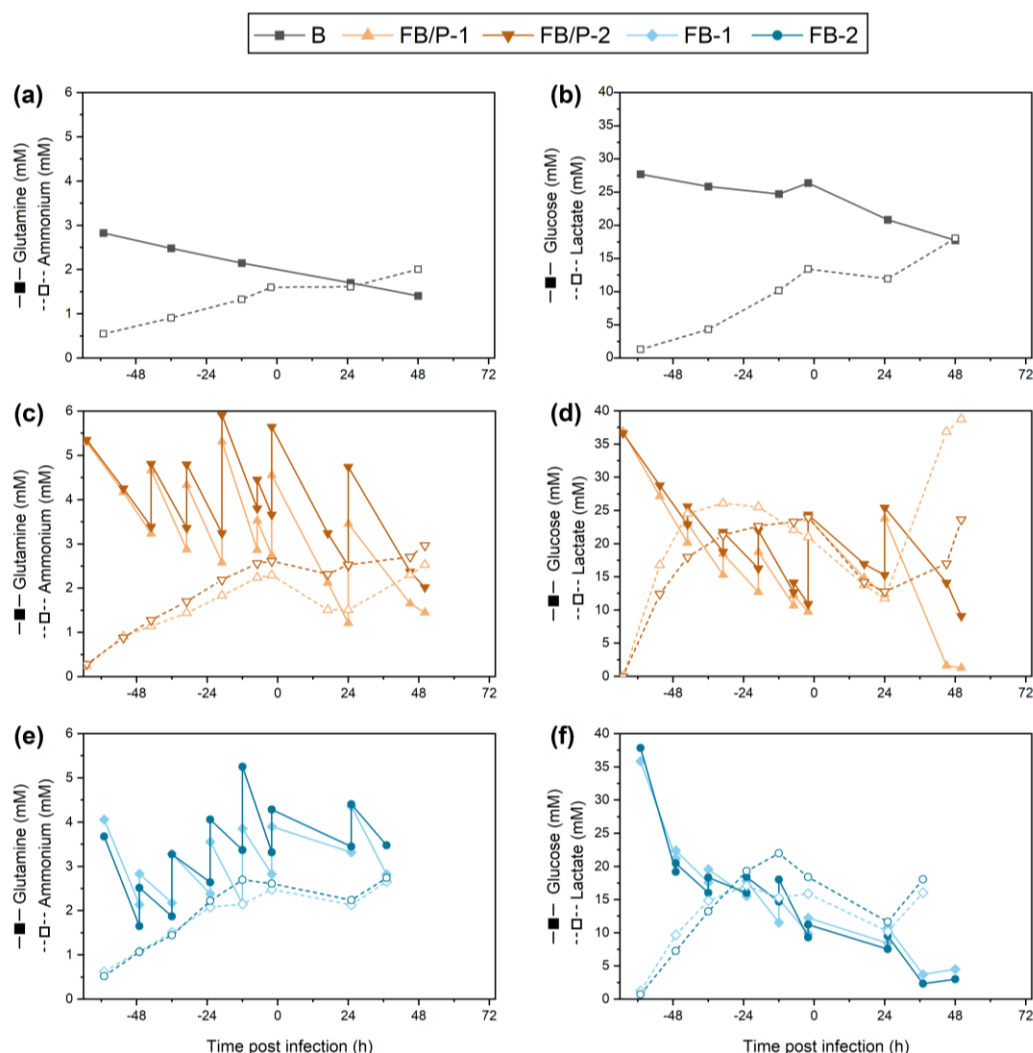


Figure 6.4 Concentration of main nutrients and metabolites in the supernatant of HEK293SF cultures producing rVSV-SARS-CoV-2 operated in batch (B, infected at 1.5×10^6 cells/mL with an MOI of 0.001), fed-batch with continuous harvest (FB/P-1 and FB/P-2, infected between 8.0 – 10.0×10^6 cells/mL with an MOI of 0.001), and fed-batch (FB-1 and FB-2, infected at 8.0×10^6 cells/mL with an MOI of 0.01). **(a, c, e)** concentrations of glutamine (full) and ammonium (empty) and **(b, d, f)** concentrations of glucose (full) and lactate (empty).

Next, a conventional fed-batch process was evaluated. Cells were seeded in fresh medium at around 2.3×10^6 cells/mL. During the growth phase, in order to evaluate the effect of different CSFR values, a volume of concentrated commercial feed corresponding to 15 pL/cell/day (FB-1) or 20 pL/cell/day (FB-2) was added every 12 hours. Cultures were infected without medium exchange at around 8.0×10^6 cells/mL with an MOI of 0.01, a 10-fold increase when compared to the batch reference, to evaluate if a faster production and therefore a shorter infection phase could be

beneficial for VSV production at higher cell densities. Following infection, concentrated feed corresponding to 5% of the culture volume was added to both cultures at 0 hpi and 24 hpi. Cells in both conditions, FB-1 and FB-2, reached a maximum of 9.5×10^6 cells/mL at 24 hpi (Figure 6.2a). Maximum virus titers of 4.5×10^{10} TCID₅₀/mL and 3.9×10^{10} TCID₅₀/mL, respectively for FB-1 and FB-2, were attained at 48 hpi (Figure 6.2b). Values of CSVY comparable to the batch reference were achieved for the fed-batch operations, respectively 4773 TCID₅₀/cell and 4074 TCID₅₀/cell for FB-1 and FB-2 (Table 6.1), demonstrating the absence of cell density effect. No meaningful difference in growth or production was observed for the two fed-batch cultivations whether feeding was performed with a CSFR of 15 pL/cell/day or 20 pL/cell/day (Figure 6.2). In fact, the concentrations of main nutrients and metabolites varied slightly for both conditions but remained at very similar levels (Figure 6.4e, Figure 6.4f). The comparable CSVY and higher titers obtained with the cultures operated in fed-batch resulted in up to 5-fold increase in Y_T (Table 6.1) and up to 5.6-fold increase in STY and VP (Figure 6.3) when compared to the batch reference.

6.7 Discussion

6.7.1 rVSV-SARS-CoV-2 production in Ambr250 bioreactors

In this study, production of rVSV-SARS-CoV-2 in a low cell density batch culture of HEK293SF cells resulted in a CSVY comparable to that attained for the same vector produced in batch cultures of suspension Vero cells (respectively 3896 TCID₅₀/cell and 3670 TCID₅₀/cell) [163]. It is also worth noting that comparable virus titers were also achieved for rVSV-ZEBOV production in low cell density batch cultures of suspension HEK293SF and suspension Vero cells [155, 163]. These results suggest that HEK293SF cells are a valuable alternative cell substrate for the production of VSV-based vectors. While the Vero cell line remains the preferred host for vaccine manufacturing due to its regulatory portfolio in this type of process, an increasing number of therapeutics produced using HEK293 cells, including the COVID-19 vaccines ChAdOx1 (Oxford-AstraZeneca) and Ad5-nCov recombinant (CanSino Biologics), are being approved and arriving in the market [153, 169-171].

In a previous work we have developed and demonstrated the efficiency of a hybrid fed-batch/perfusion process for the intensification of influenza virus production in HEK293SF cells [134]. Here, a similar process was evaluated for rVSV production. While no cell density effect was identified for influenza virus production in such hybrid mode [134], the same was not observed

here, where infections at cell densities ranging from $8.0\text{--}10.0 \times 10^6$ cells/mL resulted in up to 40% decrease in CSVY for FB/P cultures. The cell density effect has been often linked to nutrient limitation and/or accumulation of toxic by-products [23, 134, 148]. However, no clear limitation nor accumulation of by-products that could explain the drop in CSVY for both FB/P cultures was observed among the measured metabolite species. Using the same cell line employed in this study, Gobel, et al. [126] evaluated the production of a rVSV-GFP vector in perfusion. Although no limitation of quantified nutrients (glutamine and glucose) nor accumulation of by-products (ammonium and lactate) was observed, the authors reported a 40% reduction in CSVY for a perfusion culture infected at 10.0×10^6 cells/mL when compared to a low cell density batch reference, suggesting that the observed cell density effect could be linked to other factors (e.g. metabolic stress or non-quantified inhibitors). One possible explanation for the drop in CSVY observed in the present study for FB/P cultures is the longer production phase observed for rVSV production (up to 64 hpi) when compared to influenza (up to 48 hpi), which resulted in both cells and viruses being exposed to the harsher environmental conditions of a high cell density process for a longer period (high concentrations of lactate, ammonium, and other inhibitory compounds). While the use of higher MOIs for infection of low cell density cultures with VSV have been shown to have no or little effect on final titer [163](Figure 6.1), the use of higher MOIs in high cell density processes could be beneficial to minimize the exposure of cells and viruses to the cell culture environment by reducing the duration of the infection phase. In fact, no cell density effect was observed for cultures FB-1 and FB-2 – operated in fed-batch and infected at 8.0×10^6 cells/mL with a 10-fold higher MOI – despite concentrations of main nutrients and by-products comparable to FB/P cultures (Figure 6.4). These results suggest that optimal values for parameters such as the MOI, typically determined in screening studies performed in low cell density batch cultures, do not necessarily translate well when applied in high cell density processes.

No meaningful difference in cell growth and production was observed for cultures operated in fed-batch when concentrated feed addition was performed with a CSFR of 15 pL/cell/day or 20 pL/cell/day. Both conditions sustained infections performed at 8.0×10^6 cells/mL overcoming the cell density effect. These results point out to the robustness of the developed feeding regime as small variations in the volume of added feed, as well as nutrient and metabolites concentration, did not seem to impact virus production. Elahi, et al. [164] reported a fed-batch process for VSV-GFP production in high cell density cultures of HEK293SF cells, also able to overcome the cell density

effect. However, the use of in-house basal and feeding medium as well as varying compositions of medium depending on the time of feed and culture phase resulted in an overall complex process. Here we propose a simpler approach, with a chemically defined commercial feed medium added based on cell growth through a fixed CSFR.

As fed-batch processes retain the produced material inside of the bioreactor until the harvest time, the stability of the virus under culture conditions such as temperature is critical to avoid losses in infectivity in high cell density processes. Gelinas, et al. [155] evaluated the stability of a rVSV vector (rVSV-ZEBOV) when exposed to various temperatures in production medium, and reported no significant difference in infectious titers for exposures of up to 48 hours in temperatures ranging from 4°C to 34°C. In the present study, production of rVSV-SARS-CoV-2 was performed at 31°C, as this construct was designed for replication at lower temperatures to further increase the safety of the vaccine candidate [163]. Until 48 hpi (optimal harvest time) no loss in infectivity was observed for both cultures operated in fed-batch (FB-1 and FB-2). The lower temperature employed during the virus production phase might have also contributed to the stability of the virus material produced in high cell density cultures operated in fed-batch mode.

6.8 Conclusion

In this study we develop and evaluate two fed-batch-based processes to intensify the production of a rVSV-vectored vaccine candidate in suspension HEK293SF cells using Ambr250 modular bioreactors. When applied to cultures operated in fed-batch mode, the dynamic feeding strategy developed was able to support successful rVSV production in cultures infected at 8.0×10^6 cells/mL, overcoming the cell density effect and increasing total vector production in up to 5-fold compared to a batch reference. Production of rVSV in hybrid mode, on the other hand, was affected by the cell density effect in cultures infected from $8.0\text{--}10.0 \times 10^6$ cells/mL, resulting in only 4-fold increase in total vector production in comparison to the same batch reference. Due to the impact of the MOI on the duration of the infection phase, our results suggest that this parameter should be reassessed under high cell density conditions when developing intensified processes for viral vector production. Overall, our results show that fed-batch processes are a simple and effective strategy to intensify viral vector production processes, notably for vectors that are stable under normal culture conditions. Moreover, we further report HEK293SF cells as a valuable cell platform for intensified production of VSV-based vectors.

6.9 Author Contributions

Cristina A. T. Silva: Conceptualization (lead); data curation (lead); investigation (lead); methodology (lead); visualization (lead); project administration (equal); writing – original draft (lead); writing – review and editing (equal). **Amine A. Kamen:** Writing – review and editing (equal); supervision (equal); funding acquisition (lead). **Olivier Henry:** Writing – review and editing (equal); project administration (equal); supervision (lead); funding acquisition (equal).

6.10 Key points

In this article, we have evaluated fed-batch-based strategies to intensify the production of a rVSV-based vector (rVSV-SARS-CoV-2) in suspension HEK293SF cells.

A dynamic fed-batch feeding strategy, which originally consisted in the addition of concentrated feed based on cell growth and cell specific consumption of nutrients, was simplified using a fixed CSFR (pL/cell/day) factor. Addition of concentrated feed was then performed exclusively based on cell growth, eliminating the need for at line quantification of nutrients and metabolites.

The simplified dynamic fed-batch strategy was then applied to intensify the production of rVSV-SARS-CoV-2 in high cell density cultures of HEK293SF cells:

- When applied to cultures operated in fed-batch (FB), the dynamic feeding strategy was able to support rVSV production in cultures infected at 8×10^6 cells/mL, successfully overcoming the cell density effect. The high titers obtained and the low medium consumption resulted in up-to 5.6-fold increase in volumetric productivity (VP) when compared to a batch reference.
- When applied to cultures operated in hybrid fed-batch/perfusion mode (FB/P, performed semi-continuously), we observed cell density effect for cultures infected from $8\text{--}10 \times 10^6$ cells/mL.

While the use of different MOIs has been shown to not affect final virus titer in low cell density batch cultures, we hypothesize that the same is not true for high cell density cultivations. As FB cultures were infected with a 10-fold higher MOI to shorten the infection phase, the lower MOI used in FB/P cultures could be partially responsible for the observed cell density effect.

Finally, HEK293SF cells were further established as a valuable cell substrate for the production of rVSV-based vectors, presenting titers comparable or higher than those reported for suspension Vero cells.

CHAPTER 7 GENERAL DISCUSSION

As discussed in the previous chapters, the use of cell culture processes for the production of viruses is of great interest for different applications, including vaccine manufacturing and gene therapy. To keep up with the demand, cell culture processes must be intensified, which is often achieved by increasing cell concentrations while avoiding or minimizing the cell density effect. Process intensification of cell culture-based virus production has almost exclusively focused on perfusion processes [172], a type of operation that, although capable of significantly increasing production and productivity, consumes high volumes of culture medium and produces high volumes of waste [18, 21]. Fed-batch processes have been efficiently used for large-scale production of biopharmaceuticals for decades and, in addition to being well established and accepted in the industry, are able to sustain high cell concentrations with low medium consumption [16, 17, 39]. Studies over the use of fed-batch processes for virus production are very scarce. Therefore, the main goal of this doctoral research was to evaluate whether fed-batch-based feeding strategies could be adapted for influenza virus production processes, when applied in combination or not to perfusion operations.

7.1 Intensification of IAV production in HEK293 cells

7.1.1 Development of feeding strategies

In chapter 4, we have developed and presented two processes for intensified IAV production in suspension HEK293 cells. The developed processes were based on feeding strategies that minimized medium consumption and handling: a hybrid fed-batch/perfusion (also called fed-batch with continuous harvest) and a perfusion operation with a low PR.

For fed-batch operations, a dynamic feeding strategy was chosen, with concentrated feed added based on cell growth and cell specific nutrient consumption. As the cell density effect has been often linked to nutrient limitations and accumulation of toxic by-products [23, 135, 148], this feeding approach was critical to supply required nutrients and avoid overfeeding. Nonetheless, as the accumulation of rather high concentrations of by-products (lactate and ammonium) could not be avoided when reaching high cell densities at time of infection (10×10^6 cells/mL for HEK293 cells), a hybrid process was proposed: cultures were operated in fed-batch mode during the growth phase and, at time of infection, a low PR perfusion step was initiated. The continuous medium

exchange after infection alleviated by-product accumulation and provided a fresh supply of nutrients. As a result, the cell density effect was successfully overcome, and high titers of IAV could be achieved. For the perfusion operation, we selected a low PR perfusion strategy to limit the total volume of medium consumed. As our experiments, as well as available literature, had suggested a link between metabolic activity and the cell density effect [110], the perfusion strategy had to guarantee the supply of nutrients in higher cell densities despite the low PR used. For that, a higher concentration of glucose and glutamine, in comparison to basal medium, was defined for the perfusion medium. To ensure the supply of non-quantified nutrients, perfusion medium was supplemented with a percentage of concentrated feed. As a result, the low PR perfusion strategy used was able, while limiting the consumption of medium, to maintain high metabolic activity and alleviate by-product accumulation, overcoming the cell density effect and yielding high virus titers.

It is worth noticing that, in preliminary experiments, we have evaluated a hybrid process in which the steps were performed in an inverted order: a perfusion operation was performed during the growth phase and, after infection, the process was shifted to fed-batch mode. The main goal was to concentrate the virus produced during the whole infection phase in a fixed volume instead of diluting it. However, very low virus production was observed under these conditions. This is most likely due to the fast accumulation of by-products resulting from the infection at a higher cell density (9×10^6 cells/mL in these cultures) that were not removed during the infection phase. Therefore, this strategy was not further pursued.

7.1.2 Scale-up to 3 L STR bioreactor

To demonstrate the scalability of the proposed processes, developed at small scale using shake tubes, the strategies were scaled up to a 3 L STR bioreactor. The main challenge faced during the scale-up was the discrepancy in the metabolic behavior, notably for glucose and lactate, of the cells grown in the bioreactor in comparison to the shake tube cultures. Higher cell specific consumption rates for glucose combined with up to 13-fold higher $Y_{LAC/GLUC}$ resulted in lactate accumulation to potential detrimental levels (higher than 40 mM), which were not observed when the strategies were developed in shake tubes (section 4.6.3). While a number of factors can affect the metabolic behavior of cells grown in bioreactors – including DO and pH control as well as shear stress [120] – we suspect that poor CO₂ stripping, and consequent high partial pressures of CO₂ (pCO₂), could be partially responsible for the discrepancy in metabolism observed here. High pCO₂ values were

shown to impact glucose metabolism and inhibit lactate consumption in high cell density CHO cell cultures, resulting in accumulation of lactate [173, 174]. In shake tube cultures, CO₂ stripping occurs as a result of gas exchanges through the surface. Since oxygen supply also happens through the surface, low volumes of liquid (max of 20% of the total capacity of the vessel) are typically used, to ensure a high area/volume ratio and favor the transfer of O₂ and CO₂. In bioreactor cultures, on the other hand, surface aeration is often not sufficient – notably in high cell density processes – and gas transfer occurs through the sparging of air and/or pure oxygen through the liquid phase. In the bioreactor set up used for this study, oxygen supply was performed with pure oxygen sparged through a microsparger. Due to the micro-sized bubbles formed, this type of assembly is highly efficient for oxygen transfer but very little efficient for CO₂ removal [175, 176]. Additionally, because of the high oxygen transfer, very low volumes of gas were effectively sparged through the liquid. In fact, while the gas flow rate through a microsparger is typically kept between 0.005–0.01 vessel volumes per minute (VVM) [176, 177], oxygen sparging in all bioreactor runs corresponded to a rate of approximately 0.0003 VVM. This volume of sparged gas was most likely not enough to effectively strip the CO₂ produced by the cells, which probably led to high pCO₂ values. The addition of large volumes of base for pH control, observed for all high cell density bioreactor runs (not shown), could also be explained by high pCO₂ values as this would have caused a pH reduction. As the pCO₂ was not measured in the bioreactor runs, additional experiments would be required to confirm this hypothesis. The effect on cell metabolism of the addition of a macrosparger with a constant flow of air, which would increase CO₂ stripping, could be an indirect way to verify this hypothesis. The dynamic feeding strategy used during the growth phase of the hybrid fed-batch/perfusion process was able to partially accommodate this change in metabolism, and although a high concentration of lactate was attained, it did not significantly affect production. This was most likely due to the continuous harvest of virus and low residence time of particles in the bioreactor. For the perfusion operation, on the other hand, the strategy was slightly changed: instead of a fixed perfusion rate of 0.5 VVD, an increasing PR profile varying from 0.3–0.8 VVD (corresponding to an average PR of 0.5 VVD throughout the run) was used. This allowed us to reduce overfeeding in the onset of the run and increase the removal of by-products right before and after infection. As a result, lactate accumulation was minimized, and high titers of product were achieved.

When scaling up the developed processes to a 3 L STR bioreactor, we have evaluated the use of a TFDF membrane as cell retention device. Unlike other membrane-based devices, TFDF membranes make use of a depth filtration membrane (typically used for the clarification in virus production processes) that allows enveloped viruses to be harvested in the permeate line instead of retained in the bioreactor (Figure 4.11). In this study, no loss of virus nor retention was observed when using a TFDF membrane as cell retention device and harvest unit. Here, the use of a TFDF module presented two main advantages: i) allowed for the continuous harvest of the produced virus, contributing to the quality of the final product; ii) permitted that one single device performed two functions, as the harvest/clarification step – often performed using a depth filter – could be done using the same membrane and equipment. In fact, while the hybrid process required additional equipment when compared to a conventional fed-batch, the dual use of the apparatus (as cell retention device and harvest/clarification unit) could compensate the higher complexity. Other studies have reported the successful use of a TFDF module as cell retention device and clarification unit [33, 126]. Additional experiments, comparing virus material harvested directly with the TFDF and material clarified using a standard depth filter, would be required to confirm this hypothesis.

7.1.3 Outcome

Overall, the two processes developed in this chapter were able to fulfill the specific objective n°1, defined in the chapter 3:

– Specific objective n° 1 –

Develop and scale-up a process that minimizes medium consumption for intensified influenza virus production in high cell density cultures of HEK293 cells.

While the hybrid fed-batch/perfusion process is more complex to operate than a conventional fed-batch, requiring the use of a cell retention device for the perfusion step, a significant increase in productivity was achieved when compared to the batch reference. Moreover, the possible use of the cell retention device as a harvest/clarification unit could balance the additional complexity by eliminating the need of an additional depth filtration step. With the developed perfusion process, we demonstrated that perfusion strategies employing low perfusion rates are able to sustain virus production in high cell density cultures, significantly increasing process productivity when compared to a batch reference.

7.2 Intensification of IAV production in MDCK cells using fed-batch-based feeding strategies

The purpose of this doctoral research was to apply feeding strategies that minimize medium consumption to develop a generic, scalable, and efficient process for influenza virus production in mammalian cell culture. In chapter 4, while using HEK293 cells as cell substrate, we have described two intensified processes for influenza virus production. To evaluate if this type of process could be generalized to other cell lines, we have applied one of the developed operations (hybrid fed-batch/perfusion) for influenza production using a suspension MDCK cell line, the preferred host for influenza production. The suspension cell line used here had been previously reported to reach cell densities of up-to 40×10^6 cells/mL with a doubling time of around 25 h in semi-perfusion cultivations [123] and up-to 25×10^6 cells/mL in perfusion bioreactors [115]. No studies on the application of fed-batch-based feeding strategies had been previously performed using this cell line, and data on the evaluation of commercial concentrated feed medium was not available. Since this study had a limited time, being the result of a 14-week exchange program performed at the Max Planck Institute (MPI) for Dynamics of Complex Technical Systems (Magdeburg, Germany), an extensive evaluation of different commercial feeds was not feasible. Moreover, no commercial feeds developed for MDCK cells were available in the market. Therefore, we have decided to evaluate two potential commercial feeds: Hyclone Cell Boost 5 (Cytiva), due to the positive results with HEK293 cells; HEK FS (Xell/Sartorius), due to the availability of this feed medium through an ongoing collaboration from the MPI and the Xell/Sartorius.

7.2.1 IAV production in MDCK cell cultures operated in hybrid fed-batch/perfusion mode

When applying the hybrid fed-batch/perfusion strategy, developed and described in chapter 4, to MDCK cells, some compromises had to be made. Due to the limited time and the impossibility to perform extensive optimization studies, some culture methods for IAV production using this cell line were not modified from the previously established protocols developed by the MPI: i) a medium exchange step at time of infection was added to our hybrid feeding strategy, as historical data suggested that it was essential for good IAV production; ii) the PR for the perfusion step after infection was defined between 1.2 –1.5 VVD (instead of the 0.5 VVD used for HEK293 cells), as

this corresponded roughly to a CSPR of 100 pL/cell/day, half of the CSPR typically used for this cell line in perfusion bioreactors [143]. These modifications resulted in an overall higher medium consumption for the hybrid strategy when it was applied to MDCK cells. However, as this study would serve as a proof of concept for the applicability of the hybrid fed-batch/perfusion strategy for IAV production in a different cell line, these alterations were acceptable.

Preliminary experiments and historical data for influenza production using this MDCK cell line [123, 142, 143] suggested that ammonium accumulation was the major challenge for the development of a fed-batch feeding strategy. The use and maintenance of low concentrations of glutamine has been reported as an efficient approach to limit ammonium accumulation in cell culture processes [137, 140], as it restricts overflow glutamine metabolism via glutaminolysis [13, 14, 135]. Therefore, the addition of feed based on glutamine concentration and its specific consumption – approach developed and used for HEK293 cells in chapter 4 – could equally be beneficial for cultures of MDCK cells. Although glutamine concentration had to be maintained at lower levels in MDCK cell cultures (0.5 mM in comparison to 3 mM for HEK293 cells), the use of the same strategy for feed addition was capable to support cell growth and limit ammonium accumulation. The adapted hybrid fed-batch/perfusion was able to support cell growth and influenza production in cultures infected at 15×10^6 cells/mL completely overcoming the cell density effect (both in shake flasks and 3 L STR bioreactor), as well as partially overcoming the cell density effect in cultures infected at 20×10^6 cells/mL (shake flask). No virus retention nor virus loss was observed when using a TFDF membrane as cell retention device for IAV production in a MDCK culture infected at 15×10^6 cells/mL operated in hybrid fed-batch/perfusion mode.

7.2.2 IAV production in MDCK cell cultures operated in fed-batch

Due to the high growth rate of the MDCK cell line used here, we have decided to evaluate if a conventional fed-batch strategy would be able to support IAV production in high cell density cultures. Different strategies to limit/alleviate by-product accumulation were evaluated, including inoculation at higher cell densities, a medium dilution step at time of infection, as well as assessing infections performed at different cell concentrations. While the fed-batch strategies developed were able to limit ammonium concentrations below potential harmful levels, they were not able to overcome the cell density effect. Two main factors could be responsible for these results: i) while feed addition was effectively supplying glutamine and glucose, other non-quantified nutrients

(present in the basal medium but not in the concentrated feed used) were not supplied and became limiting factor for virus production; ii) although ammonium and lactate levels were under potential harmful levels, other non-quantified inhibitors (toxic metabolites, proteases) were accumulated and, as no complete medium exchange was performed, were not removed before infection. As the concentrated feed medium employed in this study was not developed for MDCK cells nor for virus production, additional studies evaluating different commercial concentrated feeds and supplementation could be performed.

7.2.3 Comparison of IAV production in hybrid fed-batch/perfusion and perfusion modes

A high cell density perfusion process, previously established at the MPI for the MDCK cell used, was performed to serve as a comparison to the hybrid fed-batch/perfusion operation. The perfusion feeding strategy consisted of a CSPR-based control of the PR, with a CSPR value of 160 pL/cell/day. As expected, the operation was able to overcome the cell density effect for cultures infected at up-to 24×10^6 cells/mL, resulting in a significant increase in virus production and process productivity. When compared to the hybrid strategy, the perfusion process was able to sustain IAV production in higher cell densities, maximizing the STY. However, a few considerations have to be made: i) when compared to the hybrid strategy, the perfusion process more than doubled medium consumption while increasing total yield of virus in 75%, which shows that medium consumption was more efficient for the hybrid strategy; ii) due to the higher volume of medium consumed, medium preparation and handling during the run was significantly more complicated for the perfusion in comparison to the hybrid operation; iii) due to the limited timeline of this study, very limited optimization studies were performed for the hybrid fed-batch/perfusion strategy, which probably resulted in larger volumes of medium consumed for the latter. The difference in volumes of medium consumed between perfusion and hybrid mode could be even higher when employing optimized concentrated feeds and protocols.

7.2.4 Outcome

Overall, the work developed in this study was able to fulfill the specific objective nº2, defined in chapter 3:

– Specific objective n° 2 –

Evaluate the developed process for intensified influenza virus production in high cell density cultures of MDCK cells.

The study demonstrated the potential of fed-batch-based processes for influenza production in MDCK cells, highlighting the need for the development of concentrated feeds specifically tailored for vaccine production processes. The applicability of the hybrid fed-batch/perfusion operation for IAV production in a different cell line was also demonstrated.

7.3 Intensification of rVSV-based vector production in HEK293SF cells using fed-batch-based feeding strategies

In chapter 4 we have described the development of efficient processes to intensify the production of IAV in HEK293 cells. As these cells are a well-established cell substrate for the production of viral vectors [30, 108, 155, 178, 179], we have decided to evaluate whether the developed feeding strategies could likewise be applied to intensify the production of a viral vector. VSV has been increasingly identified as a promising viral vector platform for vaccine manufacturing [154], with potential applications in other areas such as gene therapy and as an oncolytic virus [160, 161]. Production of VSV-based vectors to high titers has been reported for HEK293 cells [126, 155, 164], highlighting the potential of this cell line as a cell substrate. In this study, we evaluate the fed-batch-based feeding strategies developed in chapter 4 to intensify the production of a rVSV-based vaccine candidate (rVSV-SARS-CoV-2) in HEK293SF cells.

7.3.1 Definition of a CSFR to simplify feed addition

In the previous studies, described in chapter 4 and chapter 5, addition of concentrated feed during fed-batch operations was performed based on the *at line* quantification of metabolites and cell growth. Samples taken before each feeding step allowed for the estimation of the cell specific consumption/production rates for main metabolites, which in turn allowed feed to be added based on the prediction of nutrient consumption for the next time interval. During the development of the fed-batch feeding strategies, this method was critical to understand nutrient consumption, by-product formation, and the cell density effect. However, once a process is established, one should not expect large variations in the growth and the metabolic behavior of the cells as long as cultures are performed consistently (using the same cell bank, culture medium, nutrient concentrations and

protocols). In the discussion of the article presented in chapter 5, we have reported how the volumes of feed added for the same process – performed twice in shake flasks and once in bioreactor – resulted in comparable CSFRs for the different replicates (section 5.7.3). Based on this hypothesis, and since the fed-batch steps – applied before infection with the desired virus, and therefore somewhat independent of the virus used – were already established for HEK293SF cells, we have decided to evaluate if a CSFR-based feeding strategy would produce similar results of cell growth and metabolism. Based on the volumes of feed added for different replicates of the hybrid fed-batch/perfusion process for influenza production, an approximated average CSFR of 20 pL/cell/day was calculated. Therefore, for the processes presented in chapter 6, addition of concentrated feed was performed based exclusively on the cell count and growth rate according to a fixed CSFR. This simplified feeding strategy was applied to evaluate the developed hybrid fed-batch/perfusion process as well as a conventional fed-batch process. For the conventional fed-batch process, a higher MOI was defined based on the results obtained in section 5.6.3, where a higher MOI was employed to shorten the infection phase and reduce by-product accumulation during that step. Table 7.1 presents the average cell specific consumption/production rates for the main metabolites during the growth phase of cultures operated in hybrid fed-batch/perfusion mode using the original feeding protocol and the simplified approach: i) ST-FB_{HCD}, shake-tube culture for influenza virus production in hybrid fed-batch/perfusion mode infected at 8.5×10^6 cells/mL (chapter 4); ii) FB/P-1, Ambr250 culture for VSV production in hybrid fed-batch/perfusion mode, infected at 10×10^6 cells/mL (chapter 6); iii) FB/P-2, Ambr250 culture for VSV production in hybrid fed-batch/perfusion mode, infected at 8×10^6 cells/mL (chapter 6). Overall, cultures fed using the initial or the simplified strategy presented a comparable metabolic profile during the growth phase.

Table 7.1. Average specific consumption/production rates of main metabolites for cultures operated in hybrid fed-batch/perfusion mode for virus production using HEK293SF cells

Culture code	Feeding	qGLN	qNH4	qGLUC	qLAC
ST-FB _{HCD}	at line sampling	-0.59	0.25	-2.34	1.61
FB/P-1	CSFR-based	-0.60	0.20	-2.49	1.13
FB/P-2	CSFR-based	-0.60	0.25	-2.66	1.57

7.3.2 VSV production in HEK293SF cell cultures operated in hybrid fed-batch/perfusion mode

While no cell density effect was observed in cultures operated in hybrid mode for influenza production (chapter 4), the same was not true for VSV production, where a decrease of up-to 40% in CSVY was observed in hybrid cultures. As the metabolic profile of cells in both cases was comparable (Table 7.1) and no nutrient limitation nor accumulation of toxic by-products was detected for the latter, this drop in production could be due to another factor. One would expect that cultures operated in hybrid mode would perform better than conventional fed-batch cultures, as the perfusion step after infection alleviates by-product accumulation while continuously harvesting produced viruses. Interestingly, no cell density effect was observed in cultures operated in fed-batch. As no medium exchange was performed, they were infected with a 10-fold higher MOI to shorten the infection phase and limit the accumulation of toxic by-products after infection. Therefore, a possible explanation to the cell density effect observed in the hybrid cultures relates to the MOI used: it is possible that the higher MOI used in cultures operated in fed-batch was beneficial for VSV production at higher cell densities. While MOIs are typically screened in low cell density batch cultivations and applied for high cell density cultures, our results suggest that the effect of MOI may differ depending on the cell concentration. Final virus titers are greatly related to the initial concentration of virus employed as the number of synchronously infected cells in the first infection cycle as well as the activation or not of host defense mechanisms and apoptotic pathways will affect the kinetics of the infection and its success [180, 181]. Since virus replication is a complex process dependent of the host cell machinery [182-184], one can expect that the optimal MOI varies depending on the intracellular and extracellular environment, and consequently, on the cell density, metabolic state of cells, nutrient and toxic by-product concentration, etc. Additional experiments, exploring the effect of different MOIs in low and high cell density processes, would be required to confirm this hypothesis.

7.3.3 Outcome

Overall, the work developed in this chapter was able to fulfill the specific objective n°3, defined in chapter 3:

– Specific objective n° 3 –

Evaluate the developed process for the intensified production of a rVSV viral vector in high cell density cultures of HEK293 cells.

While the hybrid fed-batch/perfusion operation was not able to overcome the cell density effect when applied for VSV production in high cell density cultures of HEK293SF cells, a conventional fed-batch process performed using the same feeding strategy was able to intensify VSV production overcoming the cell density effect in cultures infected at 8×10^6 cells/mL. Moreover, we hypothesize that the use of a higher MOI could improve VSV production in high cell density cultures operated in hybrid mode.

CHAPTER 8 CONCLUSION AND RECOMMENDATIONS

8.1.1 Conclusions

The main goal of this doctoral research was to explore different feeding strategies that limit medium consumption and develop efficient processes to intensify cell culture-based influenza virus production. Due to the unique nature of virus production processes, we have built on the knowledge and techniques developed for recombinant protein production while adapting them to the virus manufacturing scenario.

As previously discussed, the intensification of cell culture-based virus production processes has almost entirely focused on perfusion techniques, and studies on the use of fed-batch operations to support virus production in HCD cultures of mammalian cells are almost inexistant. Therefore, in this work we have decided to focus on fed-batch-based and low PR perfusion operations to develop processes capable of supporting successful virus production in HCD cultures, overcoming the cell density effect. Due to the high burden of influenza viruses in worldwide health systems and the potential of cell culture processes for large scale seasonal and pandemic influenza vaccine manufacturing, we have chosen influenza as our model virus. However, due to the increasing interest over the use of cell culture processes for the production of viral vaccines and vectors, we wanted to develop generic processes that could be used with different cell lines and for the production of different viruses. The main conclusions from our investigation are presented below:

- To design strategies able to overcome the cell density effect, we first needed to understand it. Our results, as well as accumulated literature, suggest that this effect is most likely due to a combination of multiple factors including the concentration of key nutrients and by-products, the metabolic state of cells, non-quantified nutrients and inhibitors, and other parameters such as MOI. By tackling one or more of these factors, we were able to design efficient feeding strategies capable of overcoming totally or partially the cell density effect in HCD cultures. However, although the cell densities employed in this study can be considered high for virus production processes, notably in fed-batch operations, cultures with much higher cell densities are typically reported for recombinant protein production in this type of operation. The development of concentrated feed medium specifically tailored for virus production processes as well as a better understanding of the interactions

of parameters such as MOI and trypsin concentration in HCD processes would most likely allow for successful virus production in higher cell concentrations.

- One of the main challenges when developing low medium consumption feeding strategies was to supply enough nutrients to maintain cells in a desired metabolic state while limiting the formation of by-products. Since the accumulated by-products were only either partially removed (in hybrid and low PR processes) or not removed at all (conventional fed-batch processes), overcoming the cell density effect in those processes depended on finding the right balance between cell density at infection and mode of operation.
- Conventional fed-batch operations do not significantly increase the complexity of the process when compared to a batch, and even dynamic feeding strategies can be simplified through the definition of a feeding factor such as the CSFR. Therefore, this type of process has the potential to significantly increase the production of a given bioreactor without requiring important changes in the equipment. When applied for influenza production in MDCK cells, we have reported fed-batch processes that resulted in more than 3-fold increase in total production while doubling volumetric productivity when compared to the batch reference (chapter 5). When evaluated for the production of a rVSV-based vector, we have reported fed-batch processes that resulted in up-to 5-fold increase in total production and more than 5.5-fold increase in volumetric productivity in comparison to a batch reference (chapter 6). When compared to perfusion operations, this type of process could more easily be implemented in large scale, already representing a significant increase in production and productivity to a conventional low cell density batch.
- Overall, the use of hybrid operations allowed for infections to be performed at higher cell densities when compared to fed-batch processes, as the perfusion phase after infection partially removed the by-products that accumulated during the fed-batch step. When applied to influenza production in MDCK cells, the hybrid operation successfully overcame the cell density effect in cultures infected at 15×10^6 cells/mL – compared to 5×10^6 cells/mL for a fed-batch and 24×10^6 cells/mL for a perfusion process. While the need for a cell retention device increases the complexity of the operation, this type of process is a valuable alternative when medium consumption is a limitation. Moreover, we have reported that when using a TFDF membrane as cell retention device, one can couple the perfusion

step and the clarification step, typically performed by centrifugation or depth filtration, in one single device, eliminating the need for that additional step.

8.1.2 Recommendations for future work

Based on the conclusions obtained with this work, we have elaborated the following recommendations and perspectives for future work:

- The effects and the interactions of parameters such as MOI and trypsin concentration should be re-evaluated when developing high cell density processes for virus production. Moreover, as the cell density effect is most likely a result of multiple factors, one should avoid evaluating culture conditions and parameters in cultures directly inoculated at the desired high cell density, as this would eliminate the effect of the growth phase in the infection process. When evaluating culture conditions and parameters, the full process should as much as possible be taken into consideration.
- One of the challenges for the development of fed-batch operations for virus production is the lack of specific concentrated medium formulations. Therefore, collaborations with companies developing medium formulations and/or supplements for virus production processes would be highly beneficial. Additionally, although challenges with osmolarity and solubility of components should not be underestimated, the use of concentrated basal medium as feed (prepared from powder in lower volumes) could equally be evaluated.
- In this work, we have developed fed-batch feeding strategies where addition of concentrated feed was performed based on the consumption of a key nutrient (glutamine). While this approach allowed us to simplify feed addition with one single stream, it might be beneficial to separate feeding into two or more streams. A possible strategy could consist of adding a concentrated feed based on a fixed CSFR while keeping key nutrient concentrations (glutamine and glucose) in the desired levels by *at line* quantification of metabolites. This work provides some base values of CSFR for specific cell lines and medium compositions.
- In this work, the control of feed addition was performed based on the *at line* quantification of main nutrients. While the *at line* analysis of samples provides critical information on the process, the long interval between samples reduces the responsiveness of the feed control. The development of soft sensors based on *online* signals such as OUR, biocapacitance, and

fluorescence could improve the control of feed addition and increase the responsiveness and robustness of the process.

- When designing feeding strategies based on cell metabolism in small scale, one should keep in mind that some variations in the metabolic behavior of cells might occur during the scale-up process. One approach to tackle this issue is to better characterize and compare culture vessels in both scales beforehand. However, as some variation is almost inevitable, feeding strategies must be robust and able to accommodate these discrepancies.

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APPENDIX A SUPPLEMENTARY INFORMATION – INTENSIFIED INFLUENZA VIRUS PRODUCTION IN SUSPENSION HEK293SF CELL CULTURES OPERATED IN FED-BATCH OR PERFUSION WITH CONTINUOUS HARVEST

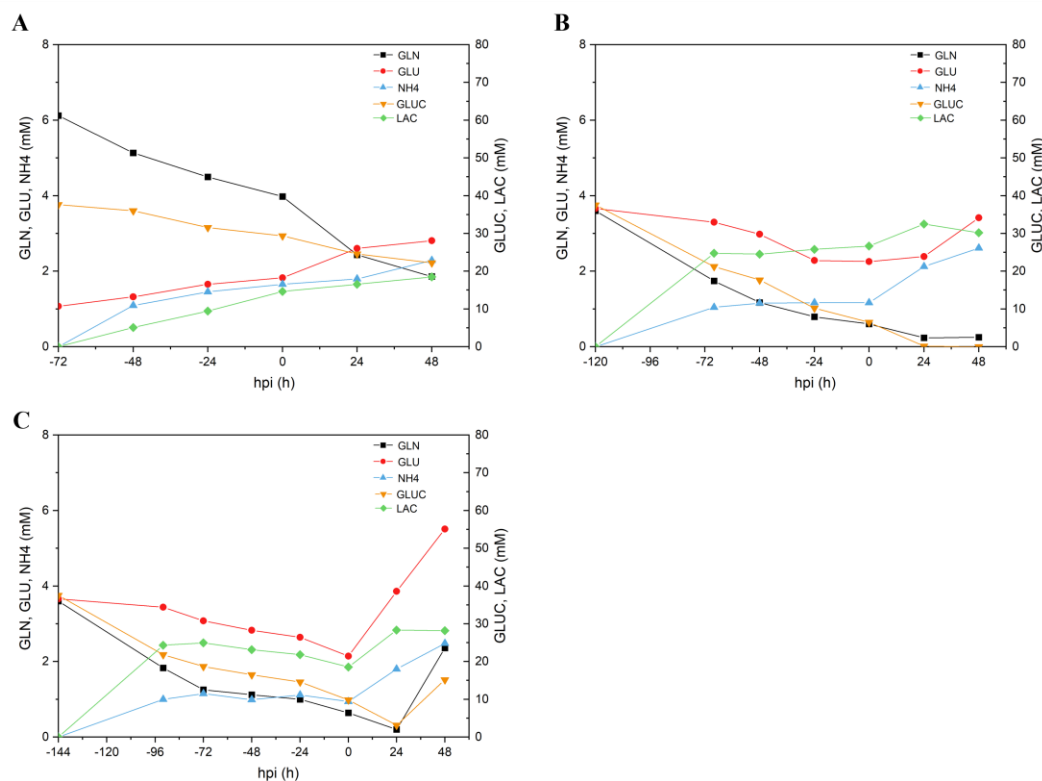


Figure A1 Nutrient and metabolite profile for 50 mL shake-tube cultures of HEK293SF cells for influenza virus production, consisting of: (A) low cell density batch (ST-B, infected at 2×10^6 cells/mL), (B) medium cell density batch (ST-B_M, infected at 4×10^6 cells/mL) and (C) high cell density 0.5 VVD pseudo-perfusion (ST-P_H, infected at 13×10^6 cells/mL). As supernatant samples were taken before media exchange or feeding, the concentration of nutrients/metabolites immediately after media exchange or feeding are not presented in the graphs.

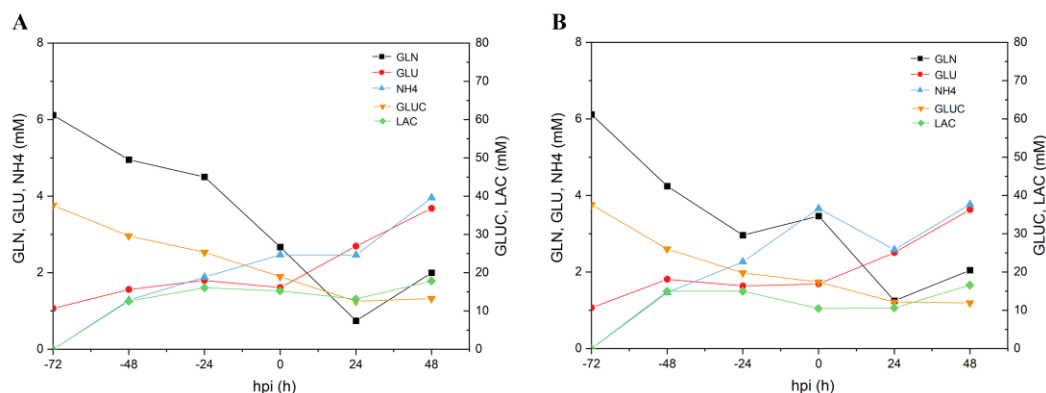


Figure A2 Nutrient and metabolite profile for 50 mL shake-tube cultures of HEK293SF cells for influenza virus production, consisting of: (A) pseudo-perfusion (ST-P_{HCD}, infected at 9.5×10^6 cells/mL), and (B) hybrid fed-batch/pseudo-perfusion (ST-FB_{HCD}, infected at 8.5×10^6 cells/mL). As supernatant samples were taken before media exchange or feeding, the concentration of nutrients/metabolites immediately after media exchange or feeding are not presented in the graph.

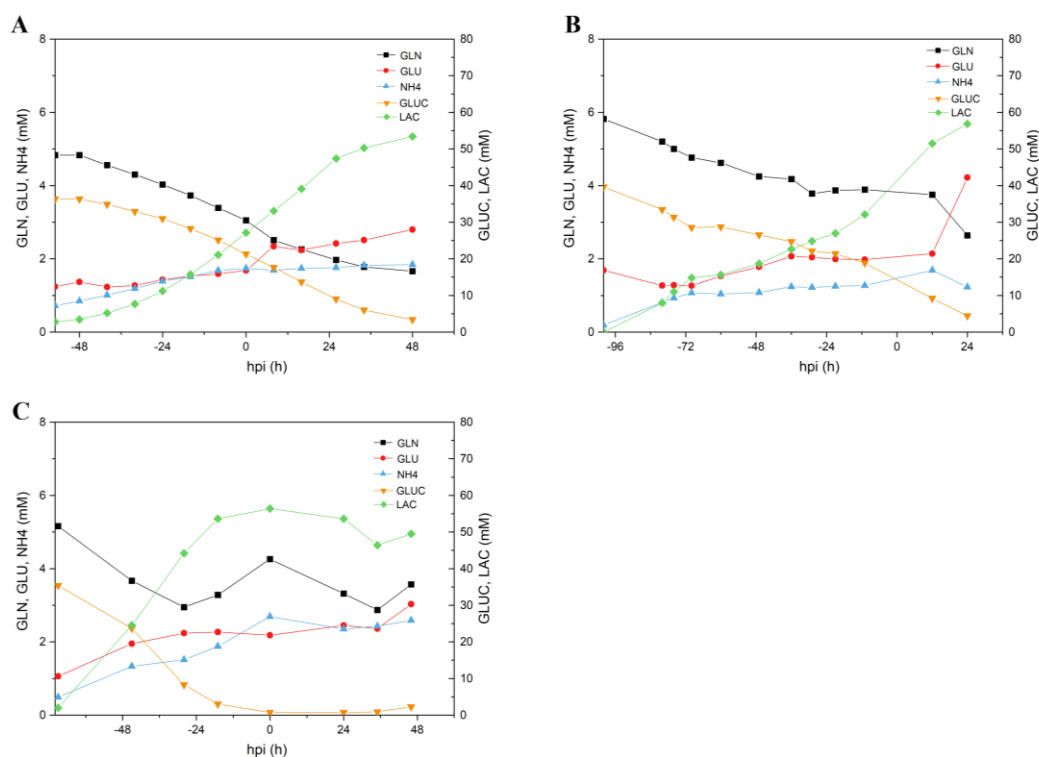


Figure A3 Nutrient and metabolite profile for 3 L STR bioreactor cultures of HEK293SF cells for influenza virus production, consisting of: (A) low cell density batch (BR-B, infected at 2×10^6 cells/mL), (B) perfusion (BR-P_{HCD}, infected at 8.5×10^6 cells/mL), and (C) hybrid fed-batch/perfusion (BR-FB_{HCD}, infected at 8.5×10^6 cells/mL).

APPENDIX B SUPPLEMENTARY INFORMATION – FED-BATCH AND PERFUSION STRATEGIES FOR PROCESS INTENSIFICATION OF MDCK CELL-BASED INFLUENZA VACCINE PRODUCTION

Table B1 Harvest volumes for each step of the final harvest process for IAV production in bioreactor cultures operated in hybrid fed-batch/perfusion and perfusion modes.

Run code	Harvest volume (mL)			
	Concentration 1	Diafiltration	Concentration 2	Total harvest pool
BR-P	610	650	300	1560
BR-FB/P	610	650	300	1560

Table B2 Average doubling time and cell specific consumption/production rates of main metabolites for shake flask and bioreactor cultures of suspension MDCK.Xeno cells during cell growth phase (average of calculated values for discrete time intervals during the growth phase).

Code	t_D (h)	q_{GLN} (pmol/cell/day)	q_{NH4} (pmol/cell/day)	q_{GLUC} (pmol/cell/day)	q_{LAC} (pmol/cell/day)	$Y_{LAC/GLUC}$	$Y_{NH4/GLN}$
B-1	18	-1.65	1.06	-2.34	2.83	1.21	0.64
P-1	21	-0.59	0.34	-1.46	1.19	0.81	0.58
FB/P-1	26	-0.58	0.25	-1.09	1.32	1.09	0.41
FB/P-2	30	-0.54	0.22	-1.06	0.98	0.82	0.39
FB/P-3	22	-0.48	0.22	-1.40	0.82	0.53	0.45
FB/P-4	22	-0.45	0.20	-1.40	0.79	0.50	0.44
FB/P-5	21	-0.28	0.09	-1.02	0.23	0.15	0.33
FB-1	18	-0.53	0.30	-1.44	1.03	0.66	0.52
FB-2	21	-0.29	0.09	-1.01	0.23	0.16	0.33
FB-3	21	-0.27	0.09	-0.99	0.24	0.17	0.32
BR-B	26	-1.32	0.75	-4.52	5.71	1.44	0.54
BR-P	24	-0.71	0.34	-2.24	1.96	0.87	0.49
BR-FB/P	24	-0.35	0.07	-1.96	1.25	0.62	0.20

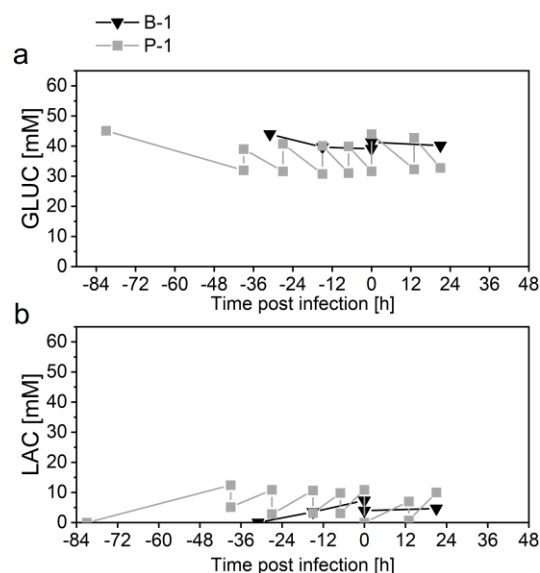


Figure B1 Extracellular concentration of **a)** glucose and **b)** lactate in suspension MDCK.Xeno cell cultures performed in 125 mL baffled shake flasks operated in batch (B-1) and semi-perfusion (P-1). Cells cultivated in batch were grown to around 3.5×10^6 cells/mL and, after a MD step, infected at 2×10^6 cells/mL. For semi-perfusion operation, a determined volume of media (corresponding to a CSPR of 100 pL/cell/day) was exchanged every 8–12 h, starting at day 2, with infection performed at 25×10^6 cells/mL.

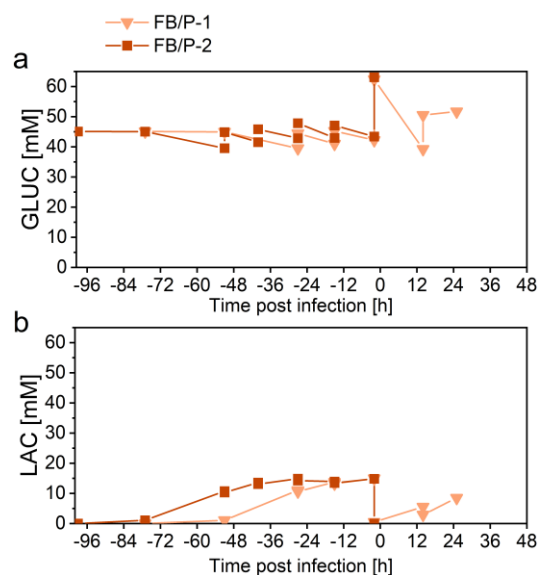


Figure B2 Extracellular concentration of **a)** glucose and **b)** lactate in suspension MDCK.Xeno cell cultures performed in 125 mL baffled shake flasks operated in FB/P, infected at 10×10^6 cells/mL (FB/P-1) and 14×10^6 cells/mL (FB/P-2). After infection, performed following a ME step, a

determined volume of media (corresponding to a PR of 1 VVD and 1.2 VVD for FB/P-1 and FB/P-2, respectively) was exchanged semi-continuously every 8–12 h.

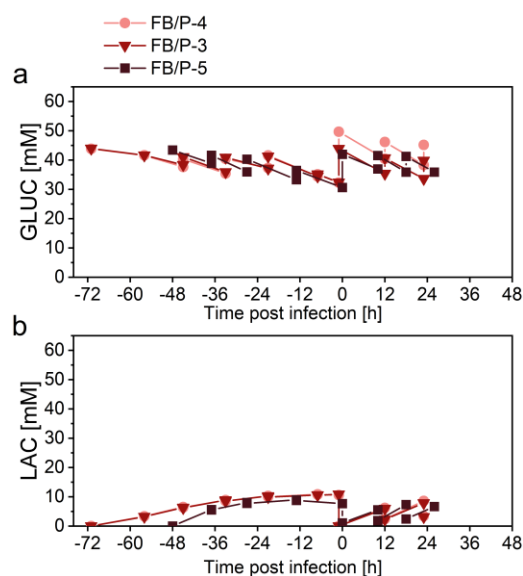


Figure B3 Extracellular concentration of **a)** glucose and **b)** lactate in suspension MDCK.Xeno cell cultures performed in 125 mL baffled shake flasks operated in FB/P, infected at 15×10^6 cells/mL (FB/P-3), 15×10^6 cells/mL with supplemented perfusion medium (FB/P-4), and 20×10^6 cells/mL. After infection, performed following a ME step, a determined volume of medium (corresponding to a PR of 1.5 VVD for FB/P-3 and FB/P-4, and 2 VVD for FB/P-5) was exchanged every 8–12 h.

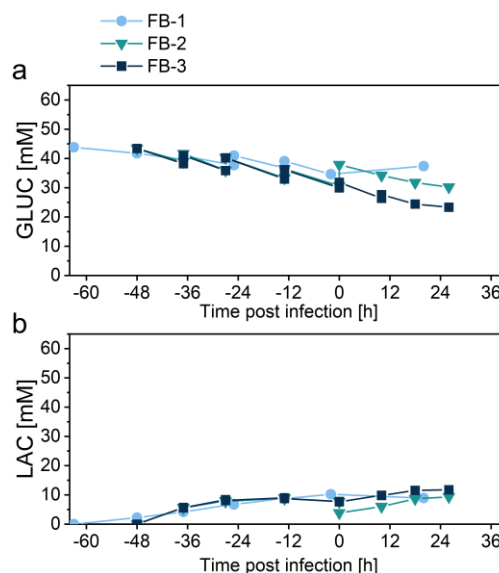


Figure B4 Extracellular concentration of **a)** glucose and **b)** lactate in suspension MDCK.Xeno cell cultures performed in 125 mL baffled shake flasks operated in fed-batch. Infection was performed

with an MOI of 0.1, at 5×10^6 cells/mL after a 1:2 MD step (FB-1), 10×10^6 cells/mL after a 1:2 MD step (FB-2), and 20×10^6 cells/mL (FB-3).

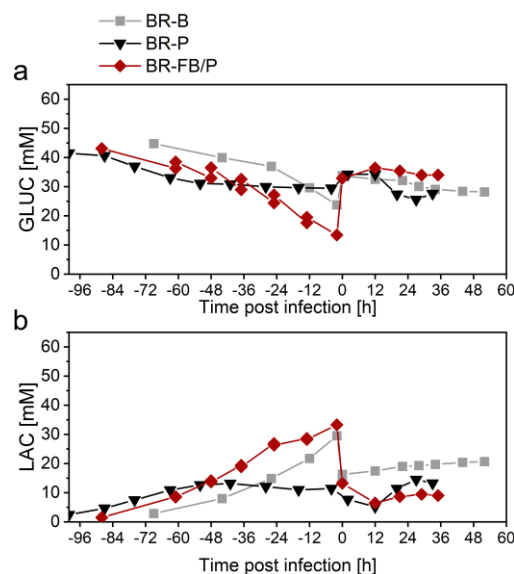


Figure B5 Extracellular concentration of a) glucose and b) lactate for 3 L STR bioreactor cultures for IAV production in suspension MDCK.Xeno cells. In the batch operation (BR-B), cells were cultured in a 1 L STR bioreactor to around 4×10^6 cells/mL and, after a MD step, infected at around 2×10^6 cells/mL. IAV production in perfusion mode (BR-P) was performed in a 3 L STR bioreactor, infected at 24×10^6 cells/mL. The perfusion was started 24 h after inoculation, and a CSPR of ~ 160 pL/cell/day was maintained through manual adjustment of the permeate flow-rate. Partial medium exchange ($1 \times V_R$) was performed at time of infection. IAV production in FB/P mode (BR-FB/P) was performed in a 3 L STR bioreactor, infected at 15×10^6 cells/mL. During the growth phase, concentrated feed was added as to maintain glutamine concentration above 0.5 mM. Partial medium exchange ($1 \times V_R$) was conducted at time of infection. Perfusion was started after infection (1.5 VVD) for the continuous harvest step.

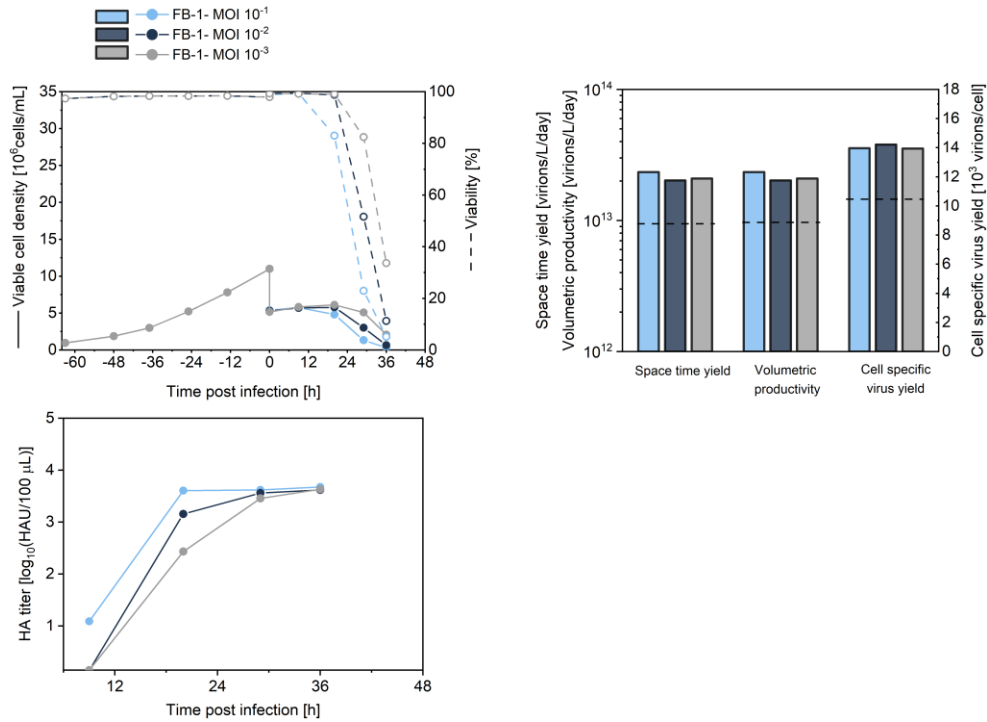


Figure B6 IAV production in suspension MDCK.Xeno cells in cultures operated in fed-batch. A single culture was grown to around 10×10^6 cells/mL (250 mL baffled shake flask) and, after a MD step, split into three 125 mL baffled shake flasks and infected at 5×10^6 cells/mL. Concentrated feed was added as to maintain glutamine concentration above 0.5 mM. Infection was performed with MOIs ranging from 0.001 to 0.1. **a)** Viable cell density (full) and viability (empty), **b)** productivity (dashed lines indicate reference values for the B-1 condition) **c)** virus concentration (HA titer).

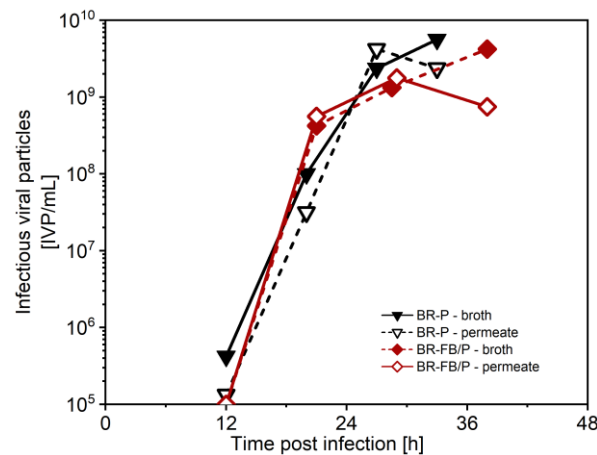


Figure B7 Infectious virus concentration (TCID₅₀) in the bioreactor broth (full symbols) and permeate line (empty symbols) for 3 L STR bioreactor cultures of suspension MDCK.Xeno cells

operated in perfusion (BR-P) and fed-batch with continuous harvest (BR-FB/P). Perfusion and continuous harvest of virus was performed using a TFDF system as cell retention device.

Table B3 Final culture volume and virus content (HA titer) before and after harvest through a TFDF membrane.

Code	Volume (mL)		Total yield (virions)	
	Bioreactor (TOH)	Harvest pool	Bioreactor (TOH)	Harvest pool
BR-P	1250	1560	1.8×10^{14}	1.8×10^{14}
BR-FB/P	1250	1560	1.1×10^{14}	8.9×10^{13}

Table B4 Values of CSFR calculated based on volume of concentrated feed added for shake-flask and bioreactor cultures operated in FB/CH infected at 15×10^6 cells/mL.

VCD (10^6 cell/mL)	CSFR (pL/cell/day)		
	FB/P-3	FB/P-4	BR-FB/P
1–9	8.9	7.9	9.2
9–13	4.8	4.9	5.7
13–16	1.6	1.5	1.8