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Influenza A virus production in a single-use orbital shaken bioreactor with ATF or TFF perfusion systems

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ABSTRACT

Driven by the concept of plug-and-play cell culture-based viral vaccine production using disposable bioreactors, we evaluated an orbital shaken bioreactor (OSB) for human influenza A virus production at high cell concentration. Therefore, the OSB model SB10-X was coupled to two hollow fiber-based perfusion systems, namely, tangential flow filtration (TFF) and alternating tangential flow filtration (ATF). The AGE1.CR.pIX avian suspension cells grew to 50×10^6 cells/mL in chemically defined medium, maintaining high cell viabilities with an average specific growth rate of 0.020 h^{-1} (doubling time = 32 h). Maximum virus titers in the range of 3.28–3.73 $\log_{10}$(HA units/100 μL) were achieved, corresponding to cell-specific virus yields of 1000–3500 virions/cell and productivities of $0.5–2.2 \times 10^{12}$ virions/L/d. This clearly demonstrates the potential of OSB operation in perfusion mode, as results achieved in a reference OSB batch cultivation were 2.64 $\log_{10}$(HA units/100 μL), 1286 virions/cell and 1.4×10^{12} virions/L/d, respectively. In summary, the SB10-X bioreactor can be operated with ATF and TFF systems, which is to our knowledge the first report regarding OSB operation in perfusion mode. Moreover, the results showed that the system is a promising cultivation system for influenza A virus vaccine production. The OSB disposable bioreactor has the potential for simplifying the scale-up from shake flasks to the large-scale bioreactor, facilitating rapid responses in the event of epidemics or pandemics.
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1. Introduction

Production processes in the pharmaceutical industry are constantly being improved to become faster, safer, more efficient and flexible, as new technologies become available [1]. Perfusion processes have been used since the 90′s for large-scale manufacturing of labile biopharmaceuticals that show fast degradation in conventional batch cultures. Nowadays, this operational mode is also used for production of a broader class of molecules including stable products (i.e., monoclonal antibodies) [2]. In perfusion, higher volumetric productivities can be achieved and product quality is often improved compared to other modes of operation [3]. In the field of virus vaccine manufacturing, a future scenario might be the establishment of high cell density perfusion processes for high titer virus production. Promising perfusion studies have been performed at lab-scale for virus vaccine production, but such processes are not yet reported for commercial vaccines. The implementation of process changes in biologics manufacturing requires time and involves significant costs. Therefore, even simple static systems are employed today for large-scale processes, which use adherent cells for virus propagation, including roller bottles and multilayer cultivation systems. The use of controlled bioreactor systems such as packed-bed bioreactors or stirred tank bioreactors (STRs) with microcarriers is also reported [4].

Following the trend towards process intensification, efforts have been made to develop designer cells for viral vaccine production that grow in chemically defined medium and in suspension, which facilitates perfusion operation. This includes, for example, AGE1.CR, AGE1.CR.pIX, CAP, EB66 and PER.C6 cells [5]. Concerning cell culture processes in general, shake flasks (Erlenmeyer) and centrifuge tubes with vented caps are widely used for screening of clones or optimization of process conditions at small-scale [6], whereas the majority of large-scale processes

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employ STR [7]. Occasionally, cultivation flasks allow for robust processes that are not easily transferred to STR. Cell growth can be limited or even impaired in STR depending on the sensitivity of cell lines to shear stress originating from stirring and bubble aeration [8]. Orbital shaken bioreactors (OSB) are a valuable alternative to STRs, as the transfer from shake flasks to OSB is simplified because these systems rely on the same basic principles for mixing and aeration and, therefore, similar hydrodynamic parameters. For this reason, scale-up in OSB can be easier, when vessels with similar geometries are used. This concerns, in particular, the adjustment of operational parameters such as shaking frequency, shaking orbit and filling volume to meet scale-up criteria (e.g. providing sufficient oxygen transfer capacity) [6,9]. In the last 15 years, many studies using orbital shaken technology were carried out using different bioreactor vessels: cylindrical, square-shaped and helical [10]. Surface aeration is an important characteristic of OSB, which results in low shear stress and reduced foam formation. For the cylindrical orbital shaken bioreactors, high volumetric oxygen transfer rates and short mixing times can be achieved depending on the bioreactor size and the shaking frequency [6,11].

Moreover, the development of single-use bioreactors rapidly increased after the first Wave bioreactor was introduced [12]. The main advantages of using single-use technology for cell culture are reduced validation and operating costs and faster turnaround between batches (no need for clean in place and steam in place procedures), besides flexibility for multiproduct facilities [13]. Examples of disposable bioreactors are: STR systems Xcellerex XDR (GE Healthcare) and S.U.B. (Thermo Scientific Hyclone), up to 2000 L in both cases; Wave bioreactors (GE Healthcare) up to 1000 L and wave-mixed bioreactors BIOSTAT® B RM (rocking motion) (Sartorius-Stedim) up to 200 L, both with maximum working volume (wv) equal to half of the total volume; and orbital shaken benchtop bioreactors (Adolf Kühner AG), up to 2500 L (wv). Wave systems are mainly used as seed-train bioreactors [14], but not so much for production compared to disposable STRs [15]. The STRs, however, involve a higher complexity regarding mounting the reactors with the integrated stirrer, while OSB enable an easier operation for large-scale bioreactors, as moving parts for stirring are not required. The latter can also contribute to lower running costs. Despite the simplicity of operation and the potential for an easier scale-up provided by OSB, studies were limited to batch and fed-batch operation [6]. To our knowledge, reports of OSB in perfusion mode are not available. Meanwhile, very high cell concentrations (10^8 cells/mL) were reached in Wave bioreactors [16], exemplifying a successful perfusion operation in a system with surface aeration.

As indicated above, virus vaccine production in lab-scale perfusion bioreactors has shown increased productivities using suspension cells in STR [17–20] and other cultivations systems, e.g. hollow fiber bioreactors [21]. In the present study, a lab-scale single-use OSB (SB10-X) was evaluated for influenza A virus (IAV) production, combining the orbital shaken technology with perfusion. As cell substrate we used the AGE1.CR.pIX suspension cell line, which grows in chemically defined medium to high cell concentration and has been previously characterized for virus propagation [18,22,23]. Here, we investigated the feasibility of OSB operation mode using either tangential flow filtration (TFF) or alternating tangential flow filtration (ATF) perfusion devices. Furthermore, IAV replication dynamics was assessed by flow cytometry, demonstrating that the cell population was sucessfully infected. Overall, we show efficient IAV production with cell-specific virus yields up to 3500 virions/cell. This represents an increase of about 2-fold compared to previous data from our lab using the same virus strain and AGE1.CR cells.

2. Material and methods

2.1. Cell line

Avian AGE1.CR.pIX suspension cells (ProBioGen AG) were cultivated in chemically-defined CD-U3 medium (Biochrom-Merck) supplemented with 2 mM L-glutamine, 2 mM alanine and 10 ng/L LONG R^3 IGF-I (Sigma). Cells were grown in baffled shake flasks at 185 rpm, 37 °C, and 5% CO_2. For passaging, cells were seeded at 0.8×10^6 cells/mL (viable cell concentration, X_v) every 3 to 4 days using 125 mL flasks (VWR, # 89095-262) with 50 mL wv. Cells were routinely subcultured until passage 100. For propagation of the inoculum, 1 L flasks (Thermo Scientific, # 4116-1000) with maximum 600 mL wv were incubated at 150 rpm. For some bioreactor cultivations, two flasks were used for inoculation.

2.2. Cultivations in the orbital shaken bioreactor

The SB10-X OSB (Adolf Kühner AG) with 12 L single-use standard bags was used for cultivations of AGE1.CR.pIX cells. Inoculation was done at $1.0–1.2 \times 10^6$ cells/mL (X_v) with 5 L initial wv. The cultivations were carried out at 37 °C with an initial shaking frequency of 70 rpm (5 cm shaking diameter). Dissolved oxygen (DO) and pH were controlled manually through adjustment of the gas composition in the output flow. Gassing flow rates (air, O_2 and CO_2) were 300–500 mL/min. The percentage of oxygen (O_2) in the gas mixture was set to 25–50% over the course of the cell growth phase to maintain the DO above 80%. The percentage of CO_2 in the gas mixture was adjusted to 0–5% during the cell growth phase for pH values above 7.2. Process parameters in reference cultivations performed in shake flasks were described by Vázquez-Ramírez et al. [18]. For virus production (Section 2.3), the percentage of O_2 in the gas mixture was increased to 40–60% and the shaking frequency was increased to 80–90 rpm.

For perfusion cultivations, either an ATF2 system with the C24U-v2 controller (Repligen) or a TFF system with a low shear centrifugal pump with magnetic levitation (Puralev® 200MU, Levitronix) was used (Fig. 1). For ATF operation, the hollow fiber (HF; Repligen, F2:RF02PES) was coupled to the bioreactor via a port

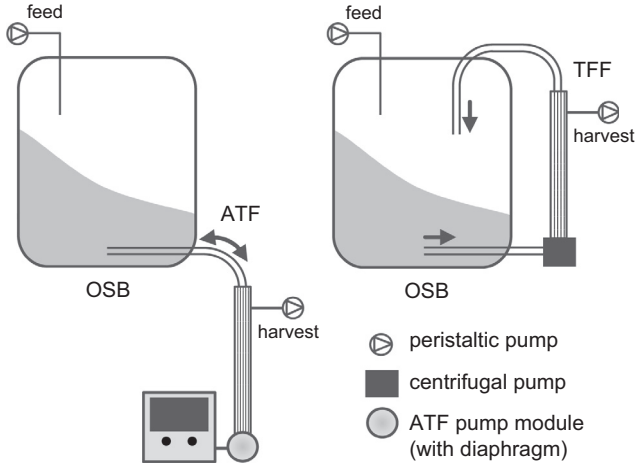

Fig. 1. Set-up used for the perfusion cultivations in the orbital shaken bioreactor (OSB). Two approaches were evaluated: either an alternating tangential flow filtration (ATF) system was coupled to the bottom of the OSB or a tangential flow filtration (TFF) unit was connected using lower and upper ports plus a centrifugal pump with magnetic levitation for recirculation. The arrows indicate the flow direction in the recirculation loop. A peristaltic pump was used for feeding and an additional pump was connected to the hollow fiber modules of ATF and TFF systems for harvesting.

located in the bottom of the bag. For TFF operation, the HF (GE Healthcare, UFP-500-E-4X2MA) was connected to the bag using a bottom port (from the bioreactor to the lower inlet of the HF) and an upper port (from the upper outlet of the HF to the bioreactor). Perfusion was started approximately 2 days after inoculation when the viable cell concentration was 3.5–5.0 × 10^6 cells/mL. Perfusion flow rates were adjusted by applying a cell-specific perfusion rate of 0.06 nL/(cell × d) to support the glucose demand [18].

2.3. Infection conditions

Avian AGE1.CR.pIX cells were infected with human influenza A/PR/8/34 H1N1 virus, derived from MCDK cells (Robert Koch Institute, Amp. 3138, $TCID_{50}$ = 1.23 × 10^8 virions/mL), with a multiplicity of infection (MOI) of 10^{-3} or 10^{-5} infectious virions/cell with addition of 10^{-7} U/cell of trypsin from a 5000 U/mL stock solution prepared in PBS (Gibco, # 27250-018). To facilitate virus uptake, perfusion was stopped for approximately 1 h after infection (by interrupting feeding and harvesting, while recirculation was maintained) and was restarted thereafter. For virus production, the bioreactor wv was increased from 5 L to 10 L (Table 1) within 24 h post infection (hpi), following a hybrid fed-batch/perfusion strategy [18,24]. In the present work, the perfusion mode continued while the dilution step was performed, instead of operating in fed-batch mode during a defined period of time and afterwards in perfusion mode. Furthermore, a second dose of 10^{-7} U/cell trypsin was administered between 14 and 24 hpi to promote multi-cycle replication, since the trypsin (26 kb) added at time of infection might be washed out in the permeate over time.

2.4. Analytical assays for cells, metabolites and virus

Viable cell concentration and cell viability were measured using a Vi-CELL® XR (Beckman Coulter) and a validated protocol for AGE1.CR.pIX cells with a maximum error (relative standard deviation) of 5% [25]. Glucose, lactate, glutamine, and ammonium (NH_4^+) concentrations were determined using a Bioprofile 100 Plus (Nova Biomedical). Measurement of virus titer was done according to standard procedures: total virus particles and infectious virus particles were measured with HA [26] and $TCID_{50}$ assays, respectively [27]. HA titers [$\log_{10}$(HA units/100 μL)] were converted into virus concentration, C_{vir} [virions/mL] assuming binding of one virus particle per erythrocyte and a given erythrocyte concentration of 2 × 10^7 cells/mL (Eq. (1)) [28].

$$C_{vir} = 2 \times 10^7 \times 10^{\log_{10}(HAunits/100\mu L)} \quad (1)$$

2.5. Imaging flow cytometry: sampling, staining and data analysis

The fraction of infected and apoptotic cells was determined by flow cytometry. Samples containing about 2 × 10^6 cells were collected twice a day after infection and fixation was performed using 2% paraformaldehyde (Morphisto GmbH) during 30 min at 4 °C. Samples were centrifuged (10 min, 300*g*, 4 °C) and the cell pellets were resuspended in 1 mL of cold PBS. Subsequently, the cell suspension was transferred to 15 mL falcon tubes containing 4.5 mL of cold 70% ethanol (v/v) and samples were stored at −20 °C until use. Staining for viral nucleoprotein (NP) and DAPI were performed similarly as described by Frensing et al. [29], but modifications in the protocol were made to minimize antibody usage and cell loss during washing steps. Blocking and antibody incubation steps were performed in 25 μL wv. After incubation steps, cells were washed two times. Subsequently, cells were resuspended in 30–50 μL FACS buffer and DAPI was added (approximately 5 μg/mL). Acquisition was performed using an ImageStream®X Mark II Imaging Flow Cytometer (Amnis, EMD Millipore); 10,000 events (single cells) were collected. Data analysis was done using IDEAS software (version 6.2). Infected cells were determined as positive for NP and apoptotic cells by analyzing DAPI signal and brightfield images.

Table 1
Experimental conditions for influenza A virus infection of AGE1.CR.pIX cells in the SB10-X bioreactor.

Run	wv[a]	MOI[b]
Batch	10	10^{-3}
ATF #1	5	10^{-5}
ATF #2	7	10^{-3}
TFF #1	8	10^{-3}
TFF #2	8	10^{-3}

[a] Bioreactor working volume in liters.
[b] Multiplicity of infection (MOI) in infectious virions per cell.

2.6. Calculations

The cell-specific virus yield (CSVY) and the culture medium productivity (P_v) were calculated from virus titers measured in the bioreactor, whereas the virus in the permeate was not considered since previous experiments demonstrated that virions do not pass through the hollow fiber membranes [23]. CSVY [virions/cell] is the ratio of the maximum virus concentration, $C_{vir,max}$ [virions/mL], and the maximum concentration of viable cells post infection, $X_{v,max}$ [cells/mL] (Eq. (2)). Because of the dilution step during virus production phase, the bioreactor working volume, wv [L] at the corresponding time points was considered, namely, t_1 = t at $C_{vir,max}$ and t_2 = t at $X_{v,max}$.

$$CSVY = \frac{C_{vir,max} * wv_{t1}}{X_{v,max} * wv_{t2}} \quad (2)$$

The culture medium productivity P_v [virions/L/d] takes into account the maximum virus concentration, the respective working volume, the total spent medium (V_{tot}) [L], and the total process time (t_{tot}) [d]considering cell growth and virus replication phases (Eq. (3)).

$$P_v = \frac{C_{vir,max} * wv_{t1}}{V_{tot} * t_{tot}} \quad (3)$$

3. Results and discussion

3.1. Single-use bioreactor operation and bag design

Perfusion cultivations were carried out using standard bags (Fig. 2-A) originally developed for batch and fed-batch processes. Because some connections were not optimal for our application, new perfusion bags were designed. The current version of the perfusion bag is shown in Fig. 2-B. Some important modifications were: (1) reduction of the tube length for connection to the perfusion systems to decrease the length of the recirculation loop; (2) integration of a diptube to enable cell addition directly into the medium, which is important for the TFF recirculation; (3) one additional perfusion connection in the bottom (two ports in total) allowing for more flexibility regarding other process options. For instance, two perfusion devices can be coupled to the OSB - one used for the cell growth phase and the other for the virus production phase. Another possibility is to use the second port as a backup, which can be relevant for industrial applications. The SB10-X bioreactor system with the standard bag is shown in Fig. 2-C.

The connection of the HF with the OSB bag is done using the bottom region of the bag (differently to the connection to a STR, done via the bioreactor headspace using a dip tube). In the first

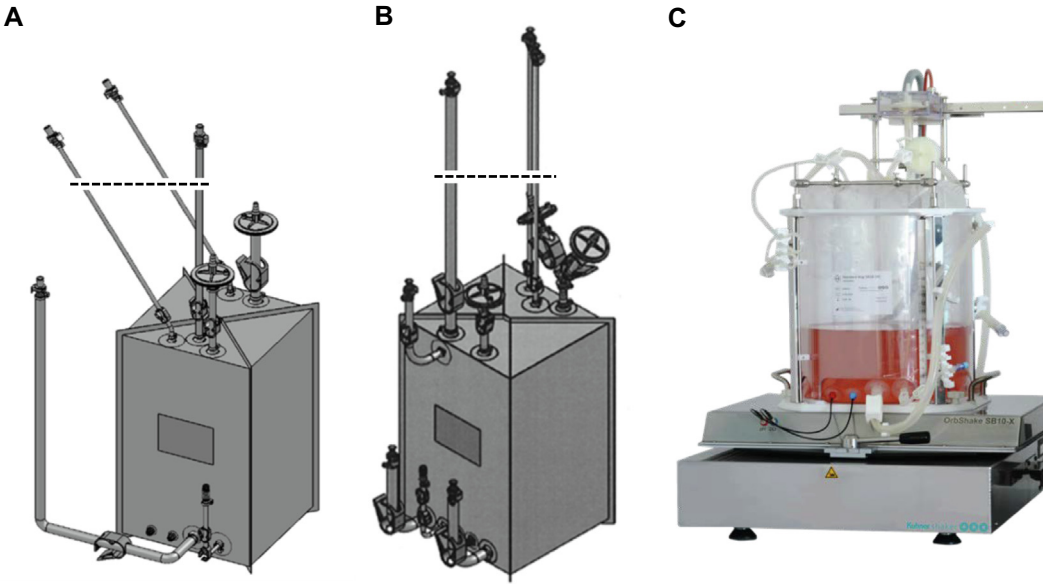

Fig. 2. Schematic drawings of single-use bags employed for cell growth and virus production in the SB10-X orbital shaken bioreactor (Adolf Kühner AG, Switzerland): (A) standard (# SMX76001) (B) perfusion (# SMX76003). Perfusion bags have two independent, short (12 cm) connections for perfusion devices in the bottom part and two exhaust air filters (connected with a Y) in the upper part. (C) Photography of the standard bag inside the vessel module, placed on top of the shaking tray.

experiments carried out at minimum filling volume (approximately 3 L), we observed air entry into the tubing connecting the bioreactor and the ATF. Because the membrane material of the HF requires operation in liquid phase and the presence of air impairs its proper functioning, the minimum volume for perfusion with ATF or TFF was increased to 5 L. Perfusion cultivations were successfully carried out at constant 5 L wv and one example (ATF #1) is shown in the results section. Aiming at a further increase in wv during the virus production phase to improve the virus yields [24], subsequent perfusion runs were carried out at 7 L (ATF #2) or 8 L scale (TFF #1 and #2). Perfusion operation at 10 L scale was also possible (not shown).

Before starting the processes, connections to the MPC couplings of the bag were done under a biological safety cabinet. To enable additional connections outside the cabinet, we used STT quick connect couplings (Sartorius Stedim) in the tubing lines (e.g., feed and inoculation), which were previously sterilized. Subsequently, about 4 L of medium was added after the bag was inflated with air (approximately 20 L of total volume). Following the time required for equilibration and subsequent calibration of the DO and pH sensors, cells and fresh medium were added to achieve the initial 5 L wv.

In the subsequent experiments, cell growth in perfusion mode was successfully carried out but an insufficient process control after infection led to low virus yields (below 1000 virions/cell). Typically after trypsin addition at TOI, an increased oxygen demand was observed for a short time (1–2 hpi), regardless of the cell line, medium and bioreactor system used. Although oxygen control via bubbling did not indicate any oxygen limitation in previous experiments, headspace aeration and current control of the SB10-X was not capable of a fast reactivity to cope with oxygen demand of cells and, in some cases, a DO drop was observed soon after infection. Therefore, the output gas mix was enriched with O_2 by manually increasing its percentage before infection. Using this configuration, virus production was enhanced (Section 3.3). After the present study was carried out, the bioreactor controller was updated regarding its response time to prevent oxygen limitation in the beginning of the virus production phase.

3.2. High cell concentration cultivations

The first cultivations were carried out to evaluate operational conditions of the newly established perfusion set-ups using ATF or TFF systems and to gain know-how regarding process control for IAV production, as discussed in the previous section. After adjusting initial parameters of each set-up, seven perfusion cultivations were successfully carried out with respect to the cell growth phase. One batch cultivation was performed for use as a reference. The success rate was higher for the ATF system compared to the TFF system due to simpler handling (e.g. a single connection for the recirculation and self-tuning of the controller). Growth of AGE1.CR.pIX cells in four representative perfusion runs and the batch control is shown in Fig. 3. Similar cell growth was obtained with the two perfusion systems and high cell concentrations were achieved with viabilities usually above 90–95%. An average cell-specific growth rate (μ) of $0.020 \pm 0.004\ h^{-1}$ (mean ± SD) was achieved in the perfusion cultivations (n = 7) considering the complete cell growth phase (from inoculation until infection). This corresponds to a doubling time (t_D) of 32 ± 7 h. The results are in agreement with previous data from perfusion cultivations in STR using AGE1.CR [23] and AGE1.CR.pIX cells [18]. For the perfusion cultivations with the best cell growth performance (e.g., ATF #2 with t_D = 27 h), viable cell concentrations above 40×10^6 cells/mL were achieved 6 days after inoculation and a maximum of 50×10^6 cells/mL one day after infection. In other cultivations, the doubling time increased considerably (up to 60%) after the start of the perfusion mode compared to the initial batch phase (e.g. ATF #1), although cultivation conditions were controlled and metabolite concentrations were maintained in the desired range. These cultures were then infected at a lower viable cell concentration to minimize medium consumption.

The concentration of extracellular metabolites of one representative perfusion cultivation and the perfusion rate used are shown in Fig. 4. During the cell growth phase, glucose levels were usually maintained above 10 mM after perfusion was started. Neither a limitation of glucose nor glutamine was observed and accumulation of lactate or ammonium did not exceed concentrations above

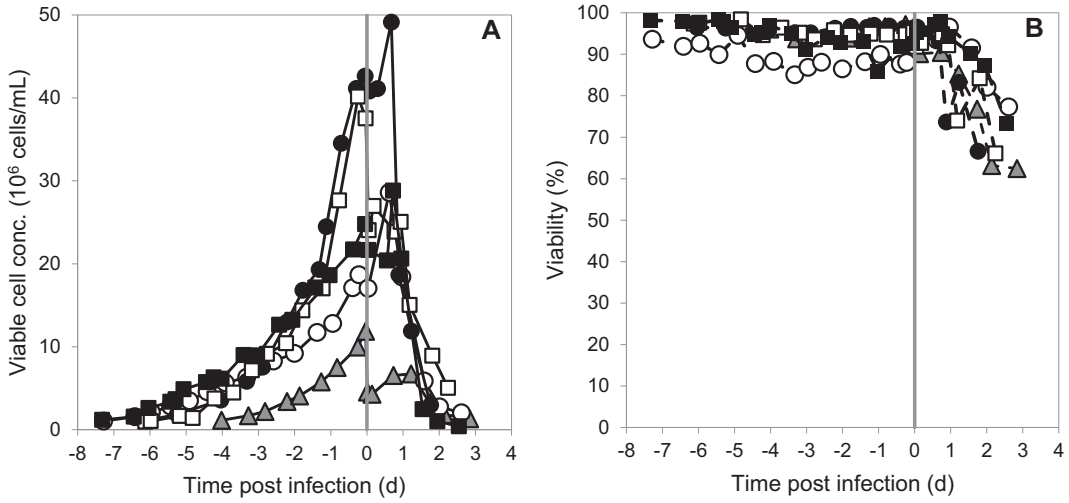

Fig. 3. Cell growth of AGE1.CR.pIX cells in CD-U3 medium in the SB10-X bioreactor (5 L initial wv) connected with ATF or TFF perfusion devices. Viable cell concentration (A) and viability (B) of one run in batch mode (▲) and four runs in perfusion mode: ATF #1 (○), ATF #2 (●), TFF #1 (□) and TFF #2 (■) (time of infection t = 0 d).

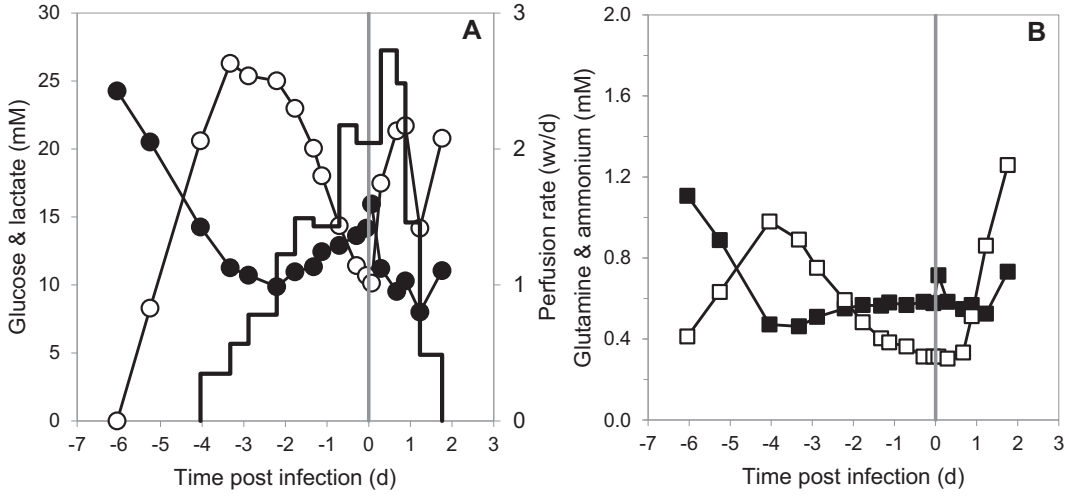

Fig. 4. Metabolite concentrations of AGE1.CR.pIX perfusion cultivation ATF #2 in CD-U3 medium carried out in the SB10-X bioreactor, subsequently infected with IAV. (A) Glucose (●) and lactate (○) concentrations; perfusion rate in the bioreactor working volume per day (wv/d) (continuous black line). (B) Glutamine (■) and ammonium (□) concentrations.

27 mM and 1 mM, respectively. Over the course of the virus production phase, ammonium concentrations increased as a consequence of cell death and release of intracellular metabolites. Nevertheless, ammonium maximum values after infection were usually 2 mM. For cell-culture based virus production, ammonium concentration in the culture can be critical. Early studies showed that high concentrations of ammonium can delay or inhibit IAV replication [30]. Furthermore, cell growth reduction after addition of ammonium salts was reported for different cell lines, while levels of toxicity varied greatly between studies (1–12 mM ammonium concentration) [31]. In the present study, the perfusion rate was increased in steps until 2–3 wv/d, to maintain the CSPR previously established for this cell line [18]. Our results confirmed that the selected strategy was sufficient to supply glucose and glutamine, while maintaining low levels of toxic by-products.

Oxygen supply can occasionally be a concern for operation at high cell concentrations [32,33]. In the present work, up to 50×10^6 cells/mL were cultivated in the bioreactor with DO values above 80% using a mixture of air and O_2 with aeration of maximum 500 mL/min (limit: 2000 mL/min). Aeration with pure oxygen, increasing volumetric rates of aeration or increasing the shaking frequency would potentially allow for cell growth to even higher concentrations. Overall, we estimated a theoretical maximum cell concentration of about 2.2×10^8 cells/mL based on Eq. (4) [34], when the volumetric mass transfer coefficient (k_La), the solubility of oxygen at saturation (C^*), the oxygen concentration in the liquid (C_L) and the cellular oxygen uptake rate (q_{O2}) are considered.

$$X_v = k_L a(C^* - C_L)/q_{O2} \quad (4)$$

For the SB10-X bioreactor, the k_La was determined for aeration with air at 1000 mL/min and shaking frequencies from 80 to 140 rpm, resulting in values of 5–25 h^{-1} for filling volumes of 8–12 L [35]. The values used for our calculations were: $k_La = 15\ h^{-1}$ (corresponding to approximately 100–120 rpm), $q_{O2} = 3.05 \times 10^{-14}$ mol/cell/h [23], $C^* = 0.60$ mmol/L for aeration with 60% of O_2 in the output flow (maximum value used in our cultivations) and $C_L = 0.16$ mmol/L for 80% of air saturation. As calculated, very high concentrations of AGE1.CR.pIX cells can theoretically be reached in an OSB with surface aeration only and without using pure oxygen. Previous cultivations of AGE1.CR cells in CD-U3

demonstrated that concentrations in the range 50–80 × 10^6 cells/mL can be achieved [18,23]. For higher cell concentrations, as obtained for CHO cells [16] or EB66 cells [17], process optimization would be required, particularly towards medium composition to support growth to very high cell concentrations while reducing the spent medium volume. Additionally, depending on the cell line and bioreactor scale, maximum cell concentrations achievable might differ, since the q_{O2} varies in a broad range (from 10^{-15} to 10^{-13} mol/cell/h for animal cell lines) [20] and k_La values tend to be lower at larger scales. Optimization studies to select key parameters such as stirring frequency and aeration strategy could be performed and process control could be improved.

3.3. Production of influenza A virus in high cell concentration cultivations

After infection with IAV at low MOI, AGE1.CR.pIX cells typically continued to grow for a short time (until approximately 1 day post infection, dpi) followed by a rapid decrease in viable cell concentration and viability, as demonstrated previously (Fig. 3-A). Perfusion rates during the virus production phase were kept higher than 2 wv/day (up to 1 dpi) and were subsequently reduced (Fig. 4-A) to follow the viable cell concentration profile. It is noteworthy that the decrease in cell concentration observed over the course of infection resulted from both the cell death and the bioreactor dilution step during the virus production phase. Volumes of 7–8 L at high cell concentration in perfusion mode worked efficiently as well as 10 L at low cell concentration in batch mode. In the TFF #1 cultivation, the bioreactor volume unintentionally increased to 9 L (1 dpi), as the membrane was partially blocked probably due to the amount of cellular debris accumulated over the course of infection. Nonetheless, the perfusion could be carried out at 9 L until the end of the infection phase.

Influenza A virus production dynamics are shown in Fig. 5-A and Fig. 5-B. The highest HA titer was 3.73 $\log_{10}$(HA units/100 μL), corresponding to 10.7 × 10^{10} virions/mL and the highest $TCID_{50}$ titer was 8.8 × 10^9 infectious virions/mL.

Regarding process control, DO in the bioreactor was maintained at high levels (>80%) until time of infection. Due to the oxygen enrichment for virus production phase, a subsequent increase (>100%) was observed, as discussed in Section 3.1. Consequently, pH levels usually increased after infection (Fig. 5-C), as no CO_2 was added in the virus production phase.

The virus concentration calculated from HA values (see Eq. (1)) was higher in OSB (Table 2) compared to previous cultivations of AGE1.CR cells in STR (1.32–6.04 × 10^{10} virions/mL), while infectious virus titers were lower than in STR (42–178 × 10^8 infectious virions/mL) [23]. In case live vaccine production is intended, further experiments are necessary to better understand IAV replication dynamics and peak infectivity in OSB. Considering that IAV vaccines are mainly inactivated vaccines, as exemplified by the cell-based vaccines approved by the FDA [36], the CSVY and the P_v were calculated based on HA (total number of virions/mL). In the best perfusion runs in OSB, CSVY up to 3500 virions/cell

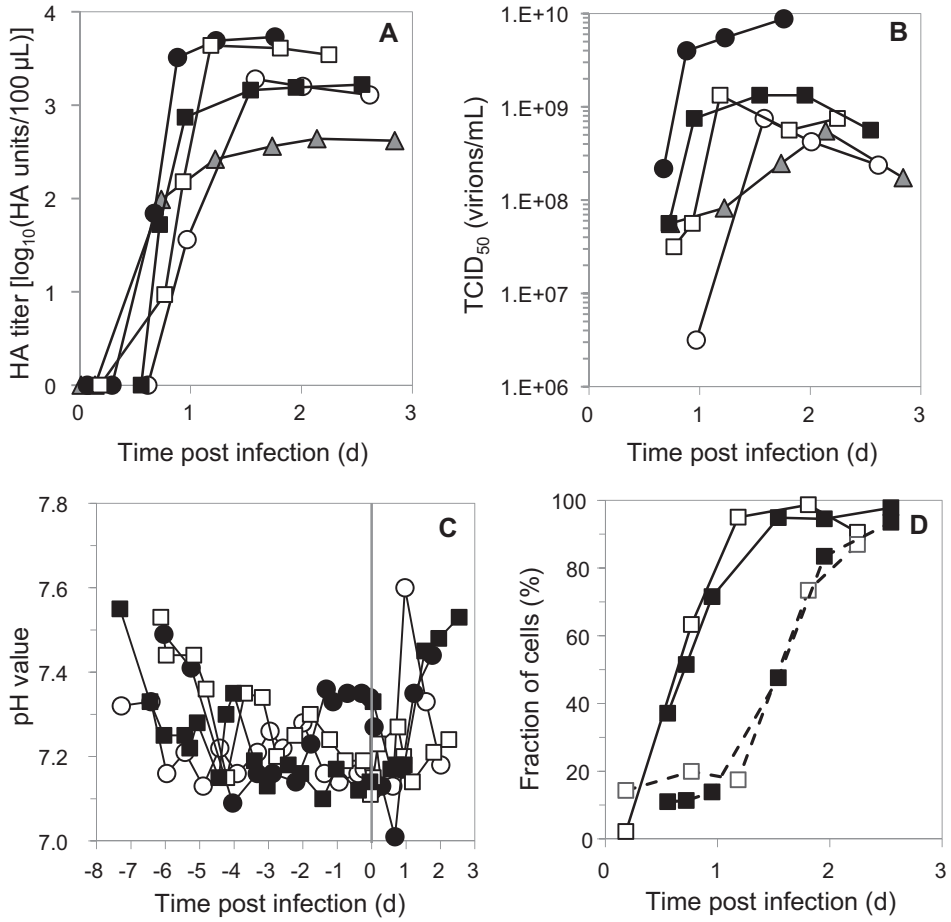

Fig. 5. Production of human influenza A virus using AGE1.CR.pIX cells grown in the SB10-X bioreactor in batch mode (▲) and in perfusion mode: ATF #1 (○), ATF #2 (●), TFF #1 (□) and TFF #2 (■) with one dilution step for virus production phase (see Table 1). (A) HA titer; (B) $TCID_{50}$ titer; (C) pH values (offline) of perfusion runs; (D) Imaging flow cytometry data for TFF #1 (□) and #2 (■): NP positive cells, infected with influenza virus (continuous line) and apoptotic cells (dashed line), showing nuclear fragmentation, based on DAPI signal and brightfield images.

Table 2
Overview of cell growth parameters and virus yields from AGE1.CR.pIX cells in OSB (wv 5–10 L).

Run	t_D[a] [h]	X_v, viab.[b] [cell/mL; %]	Max. cells[c] [$\times 10^{11}$]	Max. C_{vir}[d] [$\times 10^{10}$ v/mL]	Max. $TCID_{50}$[e] [$\times 10^{8}$ inf.v/mL]	CSVY[f] [v/cell]	P_v[g] [$\times 10^{12}$ v/L/d]
Batch	28	12×10^6, 96	0.68	0.9	5.5	1286	1.4
ATF #1	43	19×10^6, 88	1.43	3.8	7.5	1333	n.a.*
ATF #2	26	43×10^6, 96	2.46	10.7	88	3059	2.0
TFF #1	27	38×10^6, 95	2.25	8.7	13	3487	2.2
TFF #2	41	25×10^6, 93	2.39	3.3	13	1055	0.48

[a] Doubling time (t_D) during cell growth phase.
[b] Viable cell concentration (X_v) and cell viability (viab.) at time of infection.
[c] Maximum cell number after infection.
[d] Maximum virus concentration (C_{vir}) based on maximum HA titer.
[e] Maximum $TCID_{50}$ titer.
[f] Cell-specific virus yield (CSVY).
[g] Culture medium productivity (P_v).
* Medium consumption was not monitored in ATF #1.

and P_v up to 2.2×10^{12} virions/L/d were obtained. Interestingly, these results were achieved in the runs infected at higher cell concentrations with short doubling time before infection (ATF #2 and TFF #1) indicating that the better the cells grow the more virions are produced.

Furthermore, the results suggest that virus production can be improved in perfusion OSB, compared to batch OSB and to perfusion cultivations in STR with ATF [23], which yielded CSVY of 512–1708 virions/mL. The differences observed in the two bioreactor systems might be related to the response of cells to shear stress (lower in OSB) and also to the infection conditions. Particularly, the dilution step used in the present study for the virus production phase was shown to enhance virus production [18]. Further experiments are necessary to better understand how the choice of the bioreactor system impacts cell growth and consequently virus production and how the cultivation conditions can be further optimized during the virus production phase. For instance, perfusion studies for adenovirus production investigated the effect of feeding rates (0.5–3 wv/d) and the highest titer was obtained with 2 wv/d [37]. Another optimization can be towards the selection of the retention device. Currently, the preferred perfusion devices are membrane-based (ATF and TFF) because high virus titers [17,18,23] and very high cell concentrations were reported using these systems [16] and both can be used at large scale under cGMP conditions [2]. However, undesired product retention has been reported for recombinant proteins [33,38] and viruses [17,18,23]. Retention of IAV was observed using hollow fiber membranes of different materials (PS or PES) and pore sizes (50 kDa, 500 kDa, 0.2 μm and 0.5 μm) [17,18,23]. Negative effects on virus production were not observed for the larger cut-offs [23], while lower virus titers and lower cell-specific virus yield were however obtained with the 50 kDa membrane [23]. In the present work, cut-offs of 500 kDa (PS) and 0.2 μm (PES) were used and similar results were obtained. Better yields could be potentially obtained using perfusion devices that enable continuous virus harvesting, thus preventing virus degradation [39]. In addition, a disposable perfusion system would be desirable for connecting with single-use bioreactor.

Finally, to monitor the IAV replication dynamics at low MOI infection, samples from two perfusion runs (TFF #1 and TFF #2) were analyzed using imaging flow cytometry (Fig. 5-D). Almost the complete cell population (above 95%) was infected between 24 and 36 hpi indicating that the infection procedure was successfully carried out. The time point of maximum percentage of infected cells is correlated with the maximum HA values (Fig. 5-A), before the onset of cell death. Virus-induced apoptosis is a complex process [40] with different proteins modulating the course of infection and impacting the final virus titers [41,42]. In the cell-based IAV production system investigated, virus titers in the bioreactor decreased (Fig. 5-A) with an increase in the percentage of apoptotic cells (Fig. 5-D). Similar replication dynamics were observed in cultivations with adherent MCDK cells, although virus replication and release started earlier due to the high MOI used [29]. In addition, it has been reported that adherent MDCK cells cease virus release and detach from their substrate after onset of apoptosis in microcarrier cultivations [43].

4. Conclusion

For the first time, a cell-based IAV production process in an OSB coupled to perfusion devices is described. Growth of suspension AGE1.CR.pIX cells in chemically defined medium to high cell concentrations in perfusion mode (50×10^6 cells/mL) was demonstrated. High cell viabilities and short doubling times could be obtained in both TFF and ATF cultivations. Based on the cellular oxygen consumption rate of AGE1.CR.pIX cells and the oxygen transfer rate of SB10-X, cell concentrations up to 2.2×10^8 cells/mL should be feasible. Cultivations were successfully performed at 7–8 L scale in perfusion mode and at 10 L scale in batch mode, during virus production. IAV production was improved in the best perfusion cultivations compared to batch mode concerning HA titer, $TCID_{50}$ titer, CSVY and P_v. Using the OSB, the highest values reported so far for AGE1.CR.pIX cells regarding CSVY (up to 3500 virions/cell) and P_v (up to 2.2×10^{12} virions/L/d) were obtained. On the other hand, infectious virus titers obtained in OSB were lower compared to previous perfusion experiments performed in STR indicating options to further process optimization. As addressed previously, the choice of the bioreactor system is important for scale-up, especially in the case of shear sensitive cells such as AGE1.CR.pIX. Orbital shaken technology was suitable for cultivating these cells, suggesting that other animal cell lines could also be successfully cultivated in OSB in perfusion mode. Due to a simpler bag design (no stirrer, no coupling) we believe that the OSB technology could be a very competitive alternative to an STR. Furthermore, since both OSB and ATF systems are scalable, IAV production in perfusion mode could potentially be transferred from laboratory scale to production scale.

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Declaration of Competing Interest

The authors J.C., I.B., Y.G. and U.R. have no conflict of interest. T. B. and T. A. are employees of Adolf Kühner AG and V. S. is employee of ProBioGen AG, developers of the orbital shaken bioreactor and the AGE1.CR cell line, respectively.

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