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Shake tube perfusion cell cultures are suitable tools for the prediction of limiting substrate, CSPR, bleeding strategy, growth and productivity behavior

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Abstract

BACKGROUND: Scale-down models (SDM) are crucial for efficient setting of different experimental conditions in cell culture media- or clone-screening applications and to predict the expected performance in larger bioreactor cultivations. However, the availability of such models for continuous bioprocessing are scarce and hampered by the requirement for proper cell retention in perfusion applications.

RESULTS: In this study, perfusion scale-down models were optimized for increased cell performance in shake tubes by a semi-continuous operation mode without any instrumental monitoring. A 40 L perfusion bioreactor was simulated with 10 mL shake tubes by daily medium exchange with or without cell bleeding for process fine-tuning. Specifically, the optimal cell-specific perfusion rate (CSPR) was efficiently identified using the perfusion SDM. The cellular growth and productivity were nearly identical to those of the large-scale experiments. The 24-h interval of metabolite analysis identified the limits of the SDMs and underscores the need for perfusion processes in large-scale animal cell bioprocesses.

CONCLUSION: Optimized perfusion scale-down models in semi-continuous operation mode are highly suitable tools to define important process parameters of larger perfusion bioreactor cultures and are predictive of cellular growth and production behavior.
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Supporting information may be found in the online version of this article.

Keywords: perfusion; small-scale; scale-down; bioprocess intensification; continuous bioprocessing

INTRODUCTION

Perfusion bioprocesses for animal cell cultures are essential for process intensification, despite the highly complex experimental setting with additional liquid pumps and a cell retention device (Fig. 1(A)). The advantages are a high degree of automation, very high cell densities, increased volumetric productivity and space–time-yields, consistent product quality, process flexibility, and compatibility with integrated bioprocesses. A continuous medium flow at a certain cell-specific perfusion rate (CSPR in pico liter per cell per day: pL·c^{-1}·d^{-1}) provides the cells with fresh nutrients, removes toxic by-products, and enables short residence time of the protein within the bioreactor.

Optimizing a perfusion process in a holistic fashion requires multifactorial considerations, like the screening of many different conditions related to the micro- (gene, protein, cell) and macroenvironment (medium, cultivation system, process parameters). Different statistical methods, including Design of Experiment (DoE) and multivariate data analyses, support the development of a bioprocess but scale-down models (SDM) are rare. Such perfusion SDMs are limited by the extremely low liquid flow and scarcity of small scale cell retention devices.[1] Microfluidics systems with a flow rate of 7 μL·min^{-1} would be required for 10 mL working volume to reach a volumetric perfusion rate of 1 vvd. Thus, only a limited number of small-scale/miniature perfusion systems have been developed, including the Ambr™15/250 (The Automation Partnership [Cambridge] Limited),[2-6] and rocking motion (WAVE™ bioreactor from Cytiva)[7] bioreactors or the DASbox™ (Eppendorf AG) combined with ATF modules.[8] Deep-well plates, shake tubes, Erlenmeyer and spinner flasks have also been used

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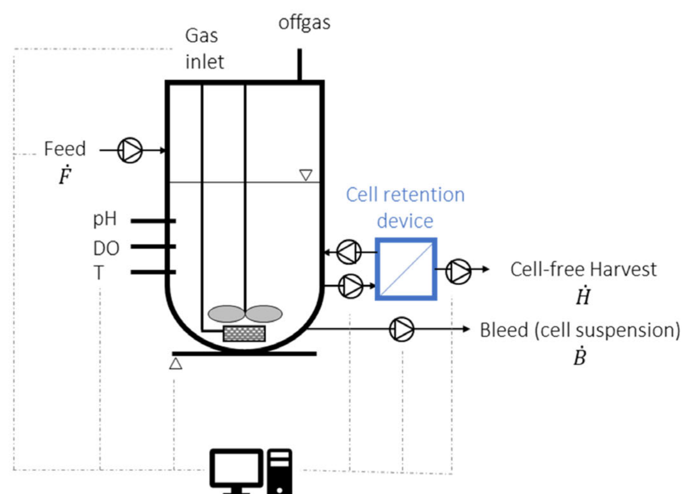

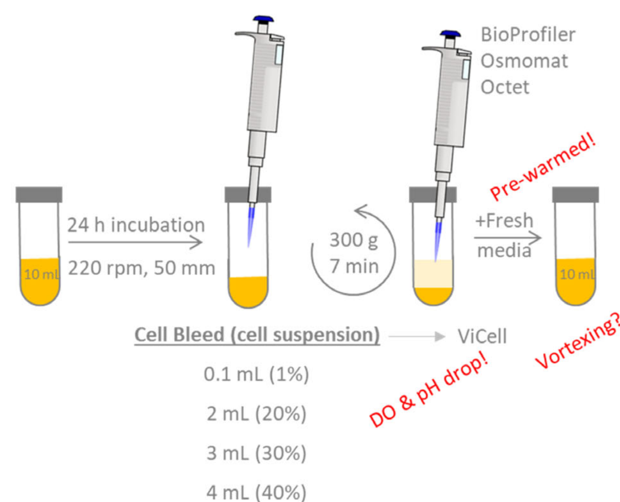

Figure 1. Comparison of a truly continuous perfusion bioreactor (A) and the optimized, non-instrumented semi-continuous scale-down (SDM) perfusion models in shake tubes (B).

as perfusion models at small scale in the past.[9-15] During perfusion medium development, SDMs are used as small surrogate cultures to estimate the 'depth'[16] of the medium (indicated by a low minimally supported CSPR), and to estimate the stability, metabolite, growth, and productivity behavior of the cell line, as factors influencing the product quality.[13]

In this study, simple non-instrumented shake tubes based on a semi-continuous centrifugation perfusion protocol were investigated for their capabilities and limitations to simulate industrial perfusion processes. Optimal shake speed, angle, working volume, and bleeding rates were defined and the methodology was used to determine the best CSPR setpoints for larger continuous perfusion bioreactors of up to 40 L. Many primary parameters of the perfusion process (CSPR, cell density, specific growth, and productivity) were accurately predicted from SDM and transferable to bioreactors. The SDM presented here predicts the growth and production behavior of perfusion cultures at different CSPRs and enables fine-tuning of the process behavior according to the specific objective (for example, N-1 seed generation, high-density cryo cell banking, steady-state production, integrated bioprocessing, or hybrid perfusion/fed-batch processes). The established method presents an inexpensive and highly effective way for perfusion cell line- and medium-screening applications.

MATERIALS AND METHODS

Cell line, media, and process analytics

A recombinant CHO-K1 cell producing a monoclonal IgG1 antibody under the GS/MSX selection system was used as model cell line throughout this study. Routine cultures were passaged twice a week at 2×10^5 c·mL^{-1} in 15 mL ActiPro™ or CDM4NS0 basal media (Cytiva) supplemented with 37.5 μmol L^{-1} MSX in Mini Bioreactor Centrifuge Tubes (Corning) with vented caps at 220 rpm, 50 mm shaking amplitude, 90° tilt angle, 37 °C, and in humid 7% CO2 atmosphere in a Kuhner shaker incubator. For initial parameter screening, a second CHO-DG44 cell line producing another recombinant IgG1 antibody under control of the dhfr/MTX selection/amplification system was used and routinely cultivated in ActiPro™ supplemented with 6 mmol L^{-1} L-glutamine and 30 nmol L^{-1} MTX. Cell counts and viability were measured on a ViCell™ XR (Beckman Coulter) instrument, metabolites on a Bioprofile™ 100 Plus (Tecom) or Cedex™ Bio (Roche), and osmolality on an Osmomat 030 (Gonotec GmbH). Titer was measured on an Octet™ Red system (Fortebio) equipped with protein A biosensors.

Semi-continuous perfusion scale-down models

Semi-continuous perfusion cultures were initiated by resuspending 10×10^6 c·mL^{-1} in 10 mL ActiPro™ or CDM4NS0-based perfusion media containing 13.94% Cell Boost™ 1 and 15.80% Cell Boost™ 3 or 11.06% Cell Boost™ 1 and 19.90% Cell Boost™ 3, respectively, in vented Mini Bioreactor Centrifuge Tubes (Corning). In addition to the conditions described above, a 45° tilt angle, shaking frequencies of 150–320 rpm, and various working volumes of 5–30 mL or incubation in 125 mL Erlenmeyer flasks were tested. Cultures were incubated for exactly 24 h after each medium exchange was executed by centrifugation at 300xg for 7 min. This was followed by a complete medium exchange. Aliquots of cell suspension were removed before centrifugation for bleeding experiments and cell count measurements. Cell-free supernatant was collected after centrifugation for measuring metabolites, titer, and osmolality. The centrifuged cell pellet was resuspended by a short, gentle vortex before initiation of the next 24 h incubation period with fresh medium. This procedure was repeated daily for 11 days.

WAVE™ bioreactor perfusion

A ReadyToProcess WAVE™ 25 system equipped with a perfusion bag containing an integrated floating filter for cell retention was used. Culture temperature was controlled at 37 °C and shaking speed was 18 rpm. The pH and DO setpoints were controlled at pH 6.8 and DO 30%. 8% sodium bicarbonate was used as base. The feed, harvest, and bleed rates were controlled gravimetrically.

XDR-50 bioreactor perfusion

An Xcellerex™ XDR-50 single-use stirred tank bioreactor (Cytiva) was used for a 40 L perfusion culture at 37 °C, 105 rpm, pH

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Table 1. Cell culture parameters for the steady-state phase of 100% retention and bled SDM in ActiPro- (day 7–11) and CDM4NS0 perfusion medium (day 5–11)

	Trend	ActiPro™ + CB1/3				CDM4NS0 + CB1/3			
Daily bleed rate (vvd)	↗	0.0	0.2	0.3	0.4	0.0	0.2	0.3	0.4
Perfusion rate (vvd)	→	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Harvest rate (vvd)	↘	1.0	0.8	0.7	0.6	1.0	0.8	0.7	0.6
VCD (MVC·mL^{-1})	↘	62	52	50	24	56	46	46	33
CSPR (pL·c^{-1}·d^{-1})	↗	14	18	19	41	16	20	21	29
Viability (%)	↗	93	97	98	98	89	94	97	98
Titer (mg·L^{-1})	↗↘	579	747	755	373	501	609	615	494
μ (day^{-1})	↗	−0.1	0.1	0.3	0.5	0.0	0.2	0.3	0.5
qP (pg·c^{-1}·d^{-1})	↗	9	16	18	20	9	15	16	20
Yield (mg)	↘	60	56	48	25	49	49	43	31
Yield with bleed recovery (mg)	↗↘	61	68	65	38	51	59	58	48
Vol. productivity (g·L^{-1}·d^{-1})	↗↘	0.56	0.63	0.57	0.25	0.47	0.51	0.46	0.35
Space–time-yield (g·L^{-1}·d^{-1})	↘	0.55	0.52	0.44	0.23	0.46	0.46	0.40	0.29
Space–time-yield with bleed recovery (g·L^{-1}·d^{-1})	↗↘	0.57	0.63	0.60	0.35	0.47	0.55	0.54	0.44
Gluc (g·L^{-1})	↗	4.8	6.1	7.5	11.5	3.8	5.9	7.1	9.6
Lac (g·L^{-1})	↘	3.4	2.9	2.5	2.1	2.7	2.5	2.2	2.0
Glu (mg·L^{-1})	↗	114	46	76	368	194	233	260	419
Gln (mg·L^{-1})	↘	172	75	49	0	221	71	11	3
NH4+ (mg·L^{-1})	↘	133	78	54	47	157	112	86	68
Osmo (mOsm·kg^{-1})	↘↗	337	317	318	351	309	297	294	310
qGluc cons. (pg·c^{-1}·d^{-1})	↗	190	222	219	268	233	262	251	274
qLac prod. (pg·c^{-1}·d^{-1})	↗	56	61	61	113	48	61	57	80
qGlu cons.(pg·c^{-1}·d^{-1})	↗↘	6.2	9.7	10.3	7.2	8.4	10.4	10.6	9.5
qGln prod. (pg·c^{-1}·d^{-1})	↘	3.0	1.7	1.2	0.0	4.3	1.9	0.3	0.1
qNH4+ prod. (pg·c^{-1}·d^{-1})	↘	1.0	0.1	−0.5	−1.5	1.8	1.3	0.7	0.4

The yield describes the mass of accumulated product since process start, the volumetric productivity describes the product mass per harvest volume per day, and the space–time-yield the yield per reactor volume and process day.

setpoint 6.8, and 40% DO limit. Xcellerex™ Automated Perfusion System (APS) (Cytiva) was used for cell retention and is based on tangential flow filtration technique (TFF). Recirculation through the hollow fiber filter (RTP CFP-4-E-9S) (Cytiva), 0.84 m^2 filter area) was achieved with a Levitronix™ single-use maglev pump (Levitronix GmbH) that is part of the single-use flow path. The shear rate in the hollow fiber filters was below 2500 s^{-1}. The pressure profile in the perfusion, such as feed, retentate, and permeate pressure, were 0.22, 0.02, and 0.13 bar, respectively.

RESULTS AND DISCUSSION

The workflow for generating a suitable small-scale model for perfusion applications starts with careful optimization of cell retention and cultivation conditions. The impact of shaking speed, tube tilt angle, and working volume were evaluated and the effect of cell bleeding strategies was investigated for a given cell line with the respective medium. Finally, the optimized small-scale models were compared to truly continuous bioreactor cultures at identical critical perfusion parameters (CSPR, along with volumetric perfusion, harvest, and bleed rates).

Shake tubes and flasks are optimal culture vessels for semi-continuous scale-down models

Efficient perfusion cultures require sufficient nutrient supply, mixing, oxygen input, and removal of toxic by-products and CO2. Additionally, a closed cell retention device (with an actual physical barrier between culture broth and complete cell-free harvest) or an open cell retention device (using physical principles for holding back most cells) is required for high cell numbers. The advantage of the open system is the removal of dead cells and cell debris from the culture broth by centrifugation, static settlers, hydrocyclones, or acoustic wave separators, and it is described as culture bleed. The recently developed mini-bioreactors represent rather expensive high-tech variants. On the other hand, shaking incubators are generally applicable for suspension cells in a volumetric range from a few to several hundred mL in deep-well plates, shake tubes, or Erlenmeyer flasks. Such cultures are not controlled for pH or DO but represent a cost-effective cultivation system for high-throughput cultivation of up to one hundred individual cultures even by a single operator. In this study, high-performing semi-continuous perfusion operation is realized by centrifugation and resuspension of the cells in completely fresh medium exactly every 24 h (Fig. 1(B)). Two different recombinant CHO cell lines were cultured in ActiPro™ basal medium in 50 mL shake tubes or 125 mL Erlenmeyer flasks at various shaking speeds and for the tubes at 90° or 45° tilt angles. Cultivation in shake tubes at 150 rpm at 90° did not provide sufficient mixing of the cells and cell numbers remained below 40×10^6 c·mL^{-1} (Supporting Information, Figure S1). Tilting the tubes to 45° restored good mixing behavior and cell numbers of up to 60×10^6 c·mL^{-1} with daily harvest titers of 0.7 g·L^{-1}. However, cultivation at 45° tilt angle led to foam formation. Especially above 200 rpm, the extensive foam formation blocked the gas-permeable membrane of the tube caps.

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The best performance was observed for Erlenmeyer flasks at various shaking frequencies or shake tubes at 90° above 200 rpm; it was identified by very high cell numbers of up to 70×10^6 $c \cdot mL^{-1}$ and daily harvest titers of 0.7 $g \cdot L^{-1}$. As shake tubes can be directly used for centrifugation, this was the culture system of choice for a semi-continuous perfusion operation. Furthermore, based on these results, a shake speed of 220 rpm and shake amplitude of 50 mm were set as the default settings for further experiments as this allows good performance of both shake tubes and Erlenmeyer flasks.

Working volume is a critical parameter to define the performance of perfusion scale-down models

The working volume of small-scale perfusion models profoundly impacts cell performance because it determines oxygen transfer and CO2 removal rates, as well as shear forces and liquid mixing behavior. Standard shaking operation conditions were defined at 220 rpm shake speed and 50 mm shaking amplitude based on the shaking parameter optimization. At this setting, a robust operation of both the 50 mL shake tubes and 125 mL Erlenmeyer flasks is possible. Reduction of the working volume in shaken tubes from 30 to 10 to 5 mL gradually increased the steady-state VCD up to 80 $MVC \cdot mL^{-1}$ together with high viability and increased daily harvest titer from 0.2 up to 0.8 $g \cdot L^{-1}$ at minimum CSPR of 12 $pL \cdot c^{-1} \cdot d^{-1}$ (Fig. 2(A)–(C)). Cultures at 10 mL initially grew fastest up to 60 $MVC \cdot mL^{-1}$, and therefore, also accumulated highest integrated viable cell days (IVCD) despite lower cell numbers. Furthermore, the cell specific productivity (qP), expressed as the slope in the cumulative titer *versus* IVCD graph (Fig. 2(D)), increased with lower working volumes despite higher VCD and lower CSPR. Even after 11 days of semi-continuous perfusion cultivation and at minimum CSPR values of 12 $pL \cdot c^{-1} \cdot d^{-1}$, viability could be maintained above 80% with suitable qP values of 10 $pg \cdot c^{-1} \cdot d^{-1}$. Increasing the shaking speed of the 5 mL culture even further to 320 rpm additionally increased cell growth but slightly lowered cell specific productivity.

Semi-continuous perfusion cultures at 30 mL entered stationary phase on day 3 at only 25 $MVC \cdot mL^{-1}$, although no nutrient depletion was observed (Supporting Information, Figure S2). Cultures with reduced working volumes consumed the nutrients provided in 24 h intervals more efficiently because of higher cell numbers and faster specific growth (Supporting Information, Figure S2). Consequently, the osmolality also decreased faster, which additionally supported faster cell growth.[17-19] The reduced growth rate at higher working volumes is probably a result of insufficient oxygen input. The oxygen input into shake cultures depends on the shaking diameter, angle, speed, oxygen concentration in the headspace, and working volume.[13,20,21] Zhu *et al.* simulated the kLa values in 50 mL orbitally shaken tubes and found good correlation with experimental kLa determination.[21] A gradual decrease of kLa with higher filling volumes and lower shaking speeds was reported. At 10 mL and 180 rpm, they reported a kLa higher than 60 h^{-1}. At 20 mL filling volume and 220 rpm, they report a kLa of around 50 h^{-1}. Furthermore, they found that, under certain conditions, kLa higher than 100 h^{-1} could be achieved, thus supporting cell densities up to 20 $MVC \cdot mL^{-1}$ by passive aeration with air. In the optimized perfusion SDM presented in our study, the cell density was even increased up to 80 $MVC \cdot mL^{-1}$. Assuming an oxygen liquid saturation concentration of 250 $pmol \cdot mL^{-1}$ in the culture broth and a specific oxygen uptake rate of 0.29 $pmol \cdot c^{-1} \cdot h^{-1}$ for the used CHO-K1 cell line,[22,23] we can calculate a minimally required kLa of 93 h^{-1}, according to Eqn (1).

$$x_{max} = kLa \times (C^* - C)/q_{02}. \quad (1)$$

x_{max} …maximum supported cell density.

C^* … liquid phase oxygen concentration at equilibrium with gas phase concentration (assumption: 8 $mg \cdot L^{-1}$ O2 = 250 $pmol \cdot mL^{-1}$).

C … (critical) dissolved oxygen concentration (assumption: 0 $pmol \cdot mL^{-1}$).

kLa … gas transfer coefficient (h-1).

q_{O2} … cell-specific oxygen consumption rate (0.29 $pmol \cdot c^{-1} \cdot h^{-1}$).

Impact of optimal bleeding rates determined by scale-down perfusion models

A separate split stream of cell suspension is often applied in perfusion cultures to introduce a certain rate of cell bleed to maintain the VCD below a critical setpoint, in addition to debris removal and the prevention of nutrient depletion, by-product toxicity, or an insufficient oxygen supply. This split stream is usually considered perfusion waste and the associated liquid supernatant (together with the cell biomass and antibody product) is discarded. Consequently, optimal perfusion processes comply with fine-tuned bleeding rates for sustained culture performance and maximized final product yields.Limiting the daily bleed rates allowed VCD to keep maximum levels of 70 $MVC \cdot mL^{-1}$ after 4–5 days and very low (critical) medium consumption rates (CSPR of 14 $pL \cdot c^{-1} \cdot d^{-1}$) at a constant medium exchange rate of 1 vvd (Fig. 3). High VCD and low product dilution translates into daily harvest titers of 0.6 $g \cdot L^{-1}$ for ActiPro™ and 0.5 $g \cdot L^{-1}$ for CDM4NS0, and a cumulative titer of 6.1 and 5.0 mg total protein per mL reactor volume in an 11-day process. However, nutrient limits and insufficient oxygen supply and CO2 removal become limiting at such high VCD combined with low provided medium per cell. Consequently, viability starts to drop, and a reduction of cell-specific productivity is indicated by the reduced slope of the cumulative titer *versus* IVCD graph (Fig. 3(C) and (F)). Too low glutamate concentrations in the glutamine-free medium is indicated by increasing ammonia concentrations as seen in the unbled cultures (Supporting Information, Figure S4(I) and (J)) and also indicated by the specific ammonia production rate (Supporting Information, Figure S5(I) and (J)) during the transition from exponential growth to steady-state stationary phase. Because of limiting glutamate concentration at this point, ammonia cannot be used efficiently for endogenous glutamine biosynthesis and it accumulates in the supernatant due to the metabolization of the previously formed glutamine.

Cell bleeding was performed by daily removal of a distinct proportion of the cell suspension (0–0.4 vvd) followed by a complete medium exchange in semi-continuous SDM. This leads to homeostasis between cell growth and nutrient supply, product formation, O2 demand, and CO2 enrichment. This strategy is highly suitable to gradually decrease the steady-state VCD values from 70 to 20 $MVC \cdot mL^{-1}$ and to accurately control the CSPR above critical values (Fig. 3). At a lower VCD but at same volumetric perfusion rate of 1 vvd, cell bleeding increased the CSPR from 14 to 41 $pL \cdot c^{-1} \cdot d^{-1}$, which also results in higher nutrient levels and lower toxic metabolite concentrations.

Higher viabilities, cell-specific growth rates, and productivities were maintained at CSPR above 20 $pL \cdot c^{-1} \cdot d^{-1}$. Bleeding at 0.2–0.3 vvd leads to cumulative harvest titers of 7 mg per mL reactor volume (Fig. 3(C) and (F)) despite lower biomass (VCD and IVCD) compared to the 100% retention culture; meanwhile, bleeding

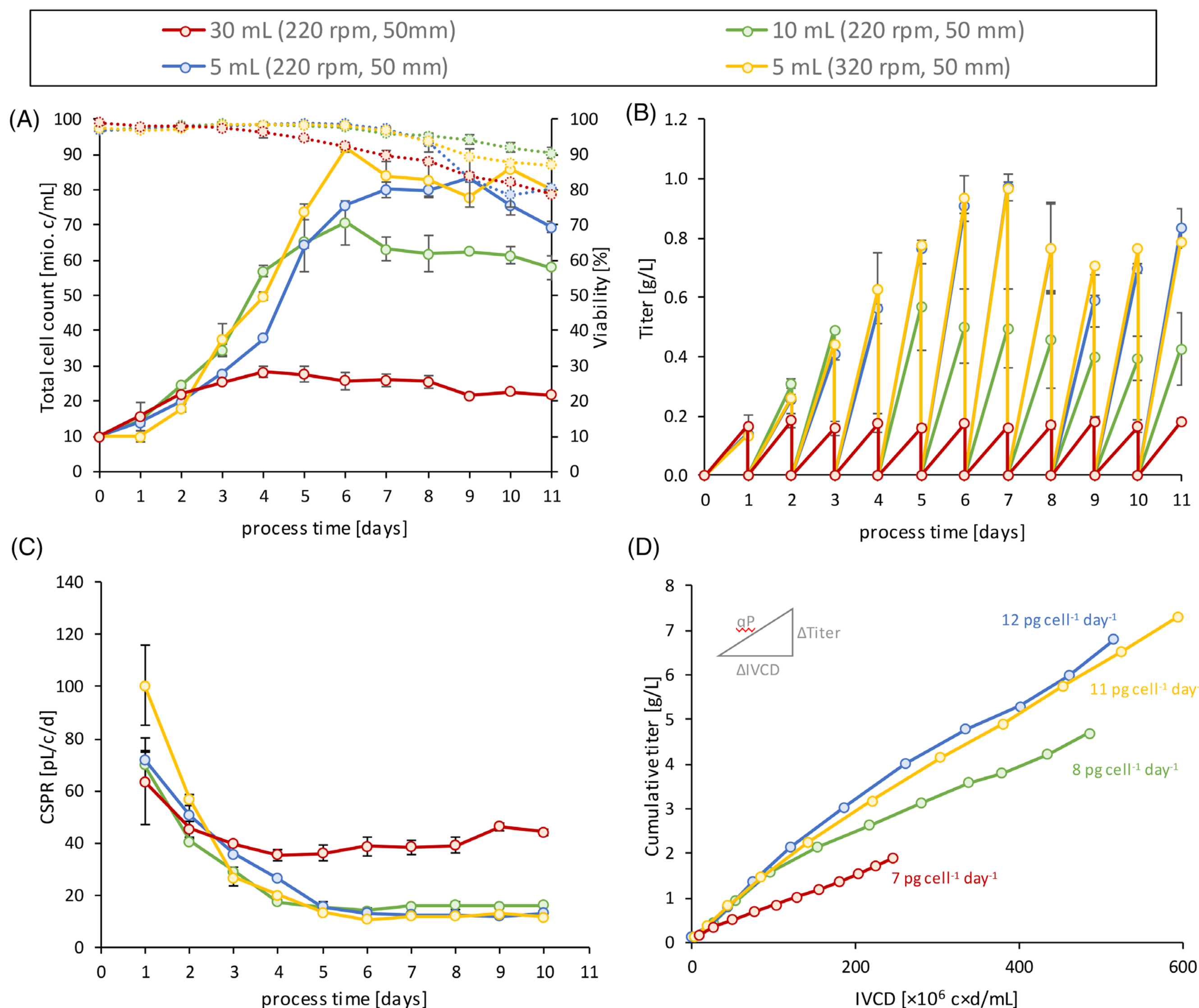

Figure 2. Effect of working volume in perfusion SDM of recombinant CHO-K1 using ActiPro™ perfusion medium. *Note:* the slope of the titer *vs.* integrated viable cell days (IVCD) graph (D) indicates the cell specific productivity, qP (pg cell^{-1} day^{-1}). Error bars are intended to indicate experimental variation of duplicate cultures and were calculated by the standard deviation.

at 0.4 vvd drastically reduced VCD, resulting in the highest cell-specific growth rate (0.5 day^{-1}). In this case, the cell-specific productivity was doubled from 10 to 20 pg·c^{-1}·d^{-1} but the final harvest titer was still reduced.

Additionally, we found that in cultures with lower VCD the ammonia concentration in the culture supernatant is much lower compared to high cell density cultures. This may have profound benefits on the product quality as ammonia is a potent inhibitor of galactosyl transferases.[24-26]

The bleeding experiments in small-scale semi-perfusion cultures were used to define the optimal operating CSPR setpoint. Figure 4 correlates the specific-growth rates and productivities in the steady-state phase with the CSPR defined by various daily bleeding rates in scale-down models. Unbled cultures with 100% cell retention showed impaired cell growth in the steady-state phase (Fig. 4(A)). By increasing the daily cell bleed, the steady-state growth rate was gradually increased to 0.2 day^{-1}, 0.3 day^{-1}, and 0.5 day^{-1} at 0.2 vvd, 0.3 vvd, and 0.4 vvd, respectively. Thus, the specific growth rate approximates the daily bleed rate. Likewise, the specific productivity was also increased at higher bleeding rates. Unbled cultures showed lowest specific productivities in the steady-state phase, and it was doubled to 20 pg·c^{-1}·d^{-1} by the cell bleed. Consequently, throughout the process, the cells maintained their productivity comparable to that of the starting level in the exponential growth phase. The final space–time-yield of the process depends on whether product can be recovered from the bled culture broth, as can be easily achieved in the SDM (Fig. 4(C)), or considered as waste (Fig. 4(D)), as is usually done in large-scale perfusion bioreactors. With the latter option, a similar STY at a 20% bleeding rate was observed for CDM4NS0 cultures compared to the unbled culture, whereas in ActiPro, higher bleeding rates led to lower final STY at the given process time. However, in all instances, bleeding significantly improved the viability (Fig. 3(C) and (F)) and provided a possibility for enhanced culture longevity and perfusion process times, both of which would finally lead to a much higher STY compared to the unbled cultures. This systematic investigation allows

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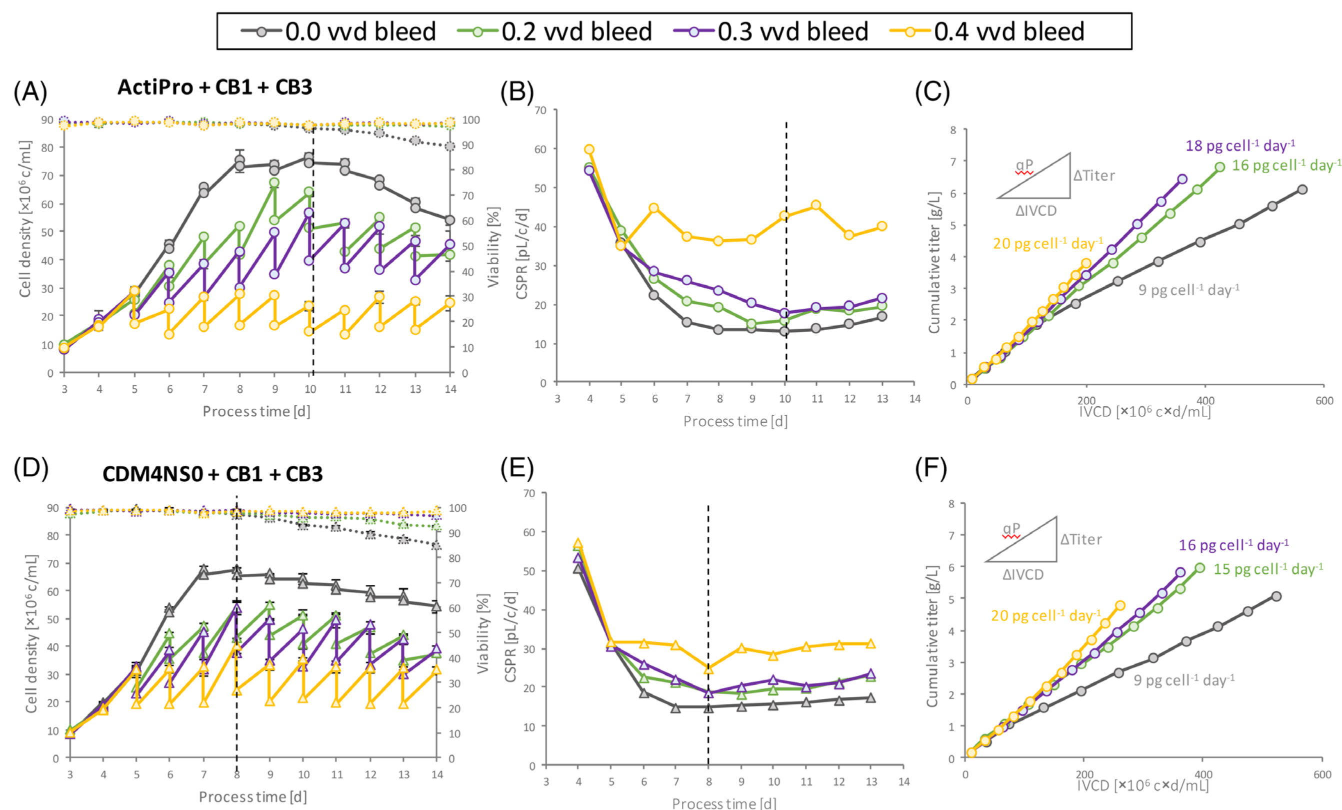

Figure 3. SDM at various daily bleeding rates in ActiPro™- (A–C) and CDM4NS0 perfusion medium (D–F). Transition from exponential to stationary (steady-state) phase is shown as a dashed line and was used to calculate average culture parameters presented in Table 1. *Note:* the slopes in the cumulative titers *vs.* IVCD graphs represents the cell-specific productivity qP (pg cell^{-1} day^{-1}). Error bars are intended to indicate experimental variation of duplicate cultures and were calculated by the standard deviation.

the definition of optimal CSPR setpoints and of objective values for truly continuous bioreactor perfusion runs. Running the small-scale models at various CSPRs identified the highest steady-state μ (0.5 day^{-1}) and qP (20 pg·c^{-1}·d^{-1}) values above 25 pL·c^{-1}·d^{-1} (Fig. 4). This CSPR limit was applied to subsequent bioreactor runs to evaluate the predictive power of 10 mL small-scale models in comparison to truly continuous 500 mL or 40 L bioreactor perfusion runs. The CSPR setpoint was chosen to achieve both a high growth and production rate. Optional CSPR setpoints can be chosen depending on the aim of the perfusion process: high growth rates are required when perfusion is used for the N-1 inoculum generation. For generating large amounts of product, however, the goal would be to limit cell growth and channel cellular energy towards maximized and maintained productivity. The small-scale models showed that cell-specific growth is limited to 0.2 day^{-1} at 20 pL·c^{-1}·d^{-1} but a high qP of 15 pg·c^{-1}·d^{-1} is maintained. Small-scale perfusion models operated at various CSPRs controlled by cell bleeds would also allow control of the metabolic cell state [e.g., higher qGluc, qLac (Supporting Information, Figure S5) and μ with higher bleeding rates], and thus, would be an ideal tool for further metabolic characterization of mammalian cell cultures.

These results highlight the importance and consequences of optimal bleeding rates in perfusion cultures at small-scale to fine-tune the available perfusion medium volume per cell (CSPR). Cell bleed, once diligently fine-tuned, represents a smart way to improve harvested product yield by means of process control at a constant feed flow without changing the medium composition. Furthermore, it allows fine-tuning of the toxic waste product concentrations and availability of nutrients; therefore, it also provides a means to control the quality of the harvested protein product.

Perfusion bleed is usually considered waste, meaning product is lost in this split stream. Consequently, various strategies have been investigated in the past to limit the growth, and thus bleed rates, of perfusion cultures by adding chemical substrates (including valeric acid or cell cycle inhibitors) or by environmental intervention with mild hypothermia to 31–33 °C.[28-30] Here, we demonstrate another possibility to limit growth while maintaining high specific productivities by carefully limiting the CSPR.

An optimal CSPR of 25 pL·c^{-1}·d^{-1} was found with this cell line/medium combination to support both high cell growth and specific productivity. Decreasing the CSPR further to 20 pL·c^{-1}·d^{-1} allows the decrease of cellular growth while maintaining productivity at a high level.

Evaluation of non-instrumented semi-continuous scale-down models in the perfusion bioreactor

The comprehensive optimization of culture parameters (including culture vessel, shake speed, medium, working volume, CSPR, and bleeding rates) was evaluated in actual continuous bioreactor perfusion runs at 40 L (Fig. 5) and 500 mL scale (Fig. 6). As cell retention device, we applied a floating filter in a rocking motion- (500 mL) reactor and a hollow-fiber tangential flow filtration (TFF) in a stirred tank (40 L) bioreactor, using the CDM4NS0 or ActiPro™ perfusion medium, respectively. A

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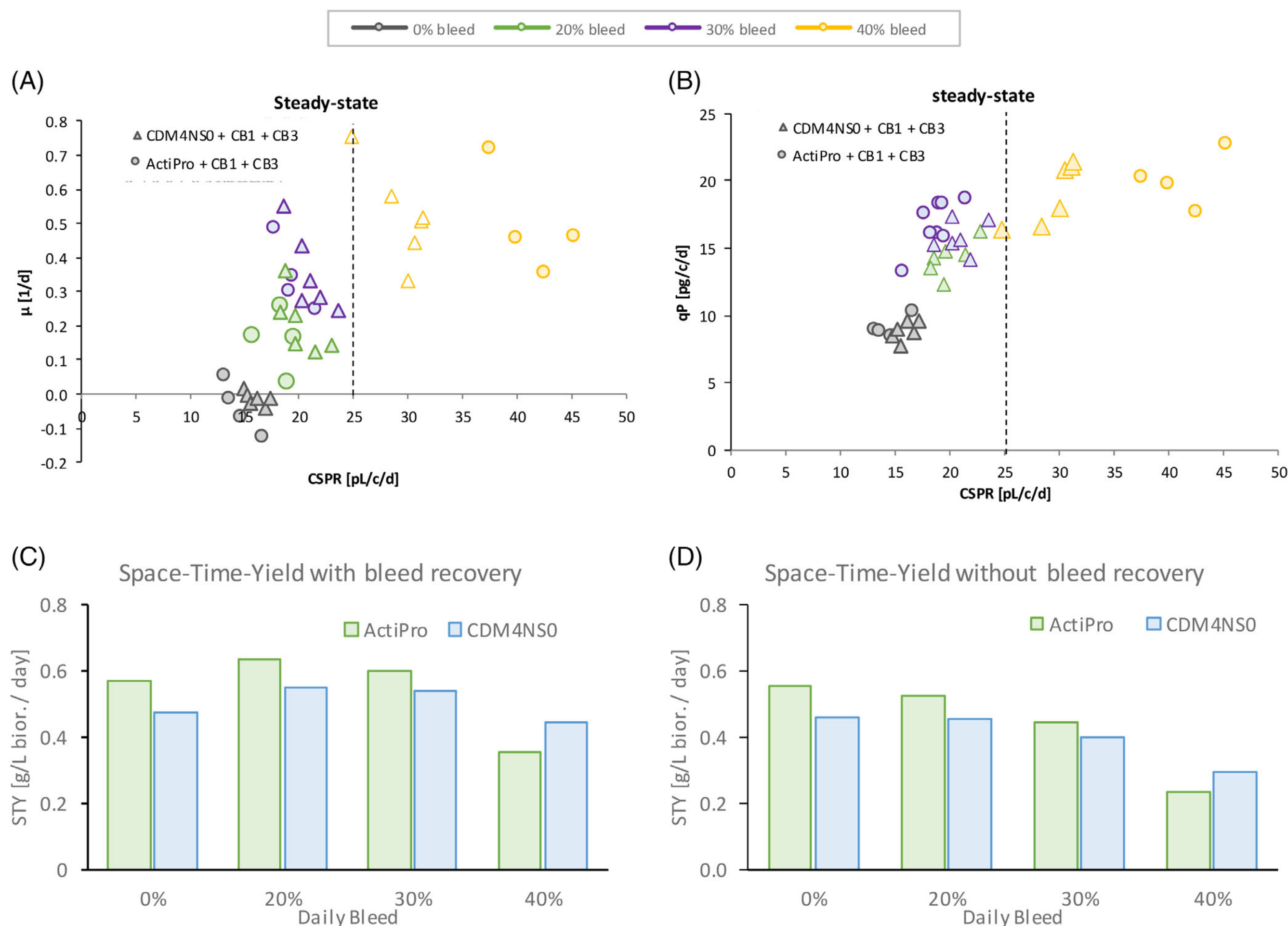

Figure 4. (A,B) Effect of bleeding on specific growth (μ) and productivity (qP) of CDM4NS0 (triangle) and ActiPro- (circles) perfusion medium. Target cell-specific perfusion rate (CSPR) of 25 pL·c^{-1}·d^{-1} (dashed line) was used for bioreactor perfusion. Higher target CSPR values are recommended for N-1 perfusion applications and lower CSPR values for steady-state perfusion to limit cell growth and bleeding rates. (C,D) Space–Time-Yields to indicate process productivity.[27] STY is increased when the bleed is recovered for product isolation because of higher cell-specific productivity. However, bleed (split) streams are often considered waste and this consequently lowers the final STY despite higher viability and specific productivity.

continuous cell bleed was introduced for the bioreactors to keep the VCD constant at 40–50 MVC·mL^{-1} and to match the daily cell bleed of 0.3 vvd applied to the semi-perfusion models.

Starting from day 6, the perfusion control parameters between the model and the 40 L ActiPro™ bioreactor were identical, allowing proper comparison of the cell performance (Fig. 5). After decreasing specific growth from 0.7 to 0.2 day^{-1}, both the SDM and bioreactor cultures reached a steady-state VCD of 45 MVC·mL^{-1} with peak titers of 0.8 g·L^{-1} measured from the culture broth on day nine. Sieving was not evaluated for the entire process, but on day 13, sieving was observed in the TFF bioreactor perfusion run with nearly 50% of the product being held back from the TFF hollow fibre. Consequently, the true cell-specific productivity is estimated to be in the order of 10 to 15 pg·c^{-1}·d^{-1} and slightly below the qP of the SDM at 15 pg·c^{-1}·d^{-1}.

Unfortunately, substantial sieving was observed in the TFF operated perfusion run. Therefore, we applied as an alternative a rocking-motion bioreactor system with an integrated floating filter device for cell retention. No sieving was observed with this setup, even at high VCD and prolonged culture time. Using the CDM4NS0 perfusion medium, highly similar CSPR values of 25 pL·c^{-1}·d^{-1} between the SDM and bioreactor could be maintained starting from day 5, which resulted in similar growth profiles at steady-state of 50 MVC·mL^{-1} and similar peak titers of 0.7 g·L^{-1} (Fig. 6). One important difference between models and bioreactors is that product is harvested continuously from the bioreactor, whereas in small-scale models, the supernatant is harvested completely once a day. In parallel, spent medium is continuously diluted with fresh medium in the bioreactor, but intermittently in SDM. Despite these important differences in the operation procedure, the small-scale models were predictive for the specific growth and productivity profile of the bioreactor. Once the cultures reached 20 pL·c^{-1}·d^{-1}, the specific growth rate decreased from (initially) 0.7 to 0.2 day^{-1} but maintained qP at 15 pg·c^{-1}·d^{-1}.

One important difference between the established SDM and upscaled continuous perfusion bioreactors is that the medium is exchanged intermittently in SDM, in contrast to a continuous perfusion flow in the bioreactor. In SDM, no probes are used to measure and control pH and DO. Furthermore, cell retention in SDM is based on centrifugation, which represents an open cell retention system were debris or protein precipitates may be removed; in the bioreactor, however, closed cell retention devices were used based on filters with defined size cut-offs. Despite these important differences, it was found that various culture parameters in

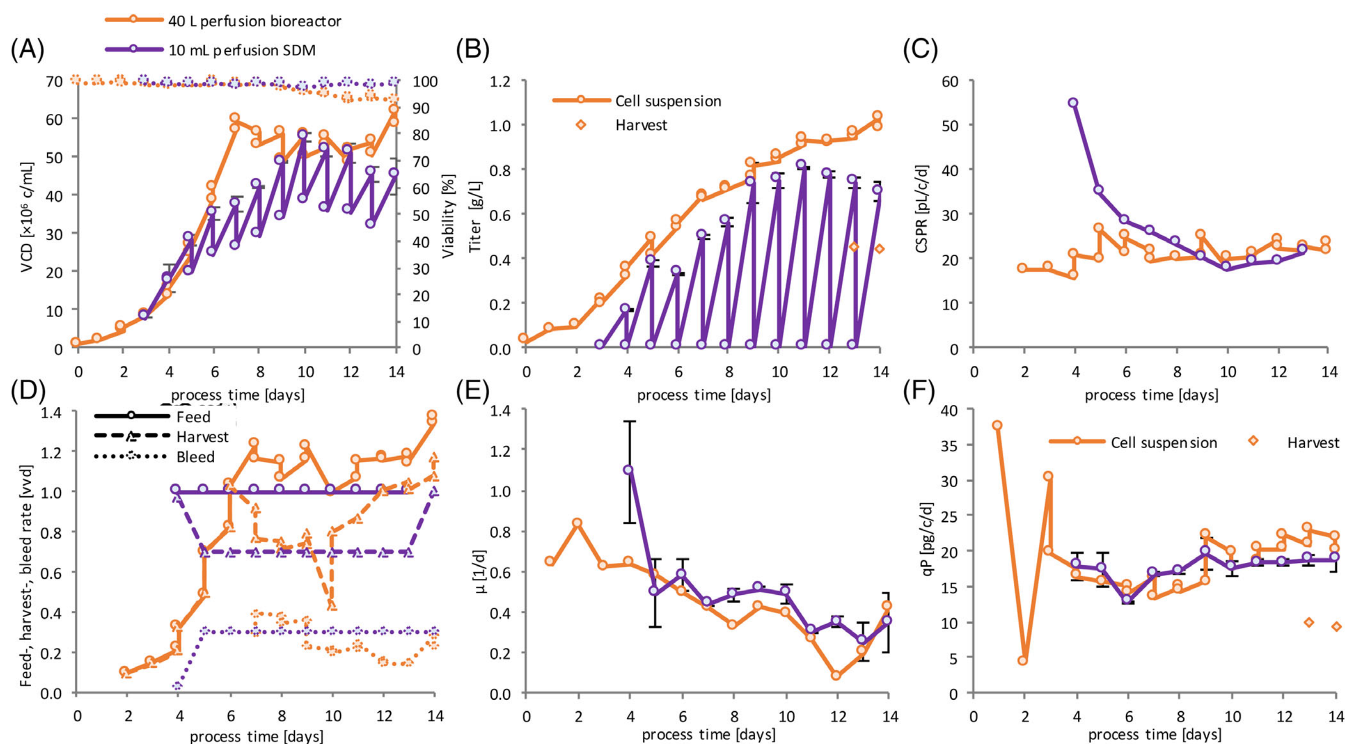

Figure 5. Comparison of SDM to 40 L perfusion bioreactor with ActiPro™ perfusion medium. To allow comparison between SDM and bioreactor culture at similar starting cell density, the starting point of SDM was set to day 3 of the bioreactor run. Error bars are intended to indicate experimental variation of duplicate cultures and were calculated by the standard deviation.

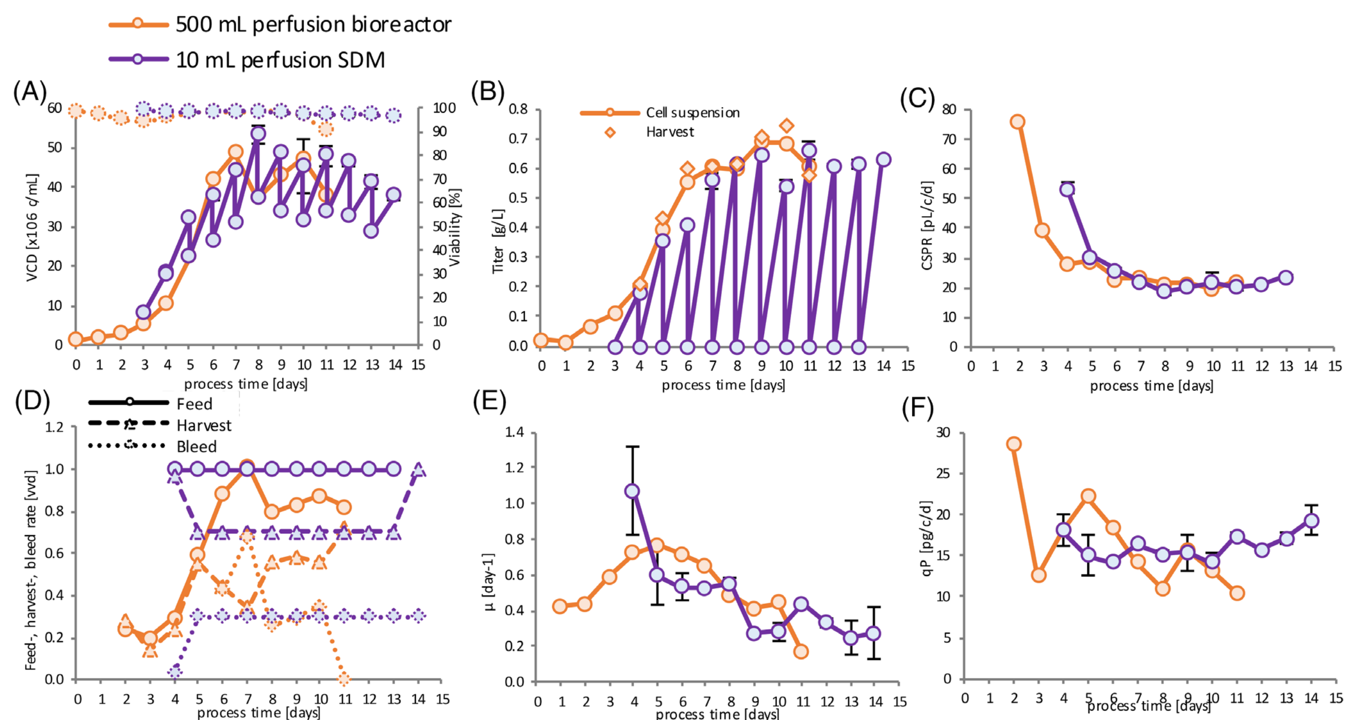

Figure 6. Comparison of SDM to 500 mL perfusion bioreactor with CDM4NS0 perfusion medium. To allow comparison between SDM and bioreactor culture at similar starting cell density, the starting point of SDM was set to day 3 of the bioreactor run. Error bars are intended to indicate experimental variation of duplicate cultures and were calculated by the standard deviation.

the SDM are predictive for scaled-up bioreactor cultures once the critical cell-specific perfusion rate of the bioreactor matches the CSPR of the SDM (Table 2).

Especially, growth and productivity profiles are well represented by the small shake tube cultures, allowing for the estimation of space–time-yields of larger bioreactor cultures.

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Table 2. Comparison of non-instrumented semi-continuous perfusion SDM and perfusion bioreactor at 40 L and 500 mL scale. A more than 50% difference of the SDM versus the 40 L bioractor is indicated in red, differences lower than 50% in green.

	SDM (Acti + CB1/3)	Bioreactor (XDR-50)	ratio SDM/40 L	SDM (NS0 + CB1/3)	Bioreactor (WAVE™ 25)	ratio SDM/500 mL
Perfusion mode	Semi-perfusion	VCD const.		Semi-perfusion	VCD const.	
Vessel	ST50	XDR-50		ST50	WAVE™25	
Basal media	ActiPro™			CDM4NS0		
Supplement	Cell Boost™ 1 + 3			Cell Boost™ 1 + 3		
Period (day)	6–14			6–14	6–11	
pH control	None (cons. 7% CO2)	Setpoint pH 6.8 (CO2/Base)		None (cons. 7% CO2)	Setpoint pH 6.8 (CO2/Base)	
DO control	None (const. 21% O2)	40% (O2, F, rpm)		None (const. 21% O2)	30% (O2, F)	
T control	37°C			37°C		
Stirring (rpm)	220 (50 mm)	105		220 (50 mm)	18	
Working volume (mL)	10	40 000		10	500	
Perfusion rate (vvd)	1.0	1.2	0.9	1.0	0.9	1.1
Harvest rate (vvd)	0.7	0.9	0.8	0.7	0.5	1.3
Bleed rate (vvd)	0.3	0.3	1.2	0.3	0.3	0.9
VCD ($MVC \cdot mL^{-1}$)	46	55	0.8	45	43	1.1
Viability (%)	98	95	1.0	98	97	1.0
Titer ($g \cdot L^{-1}$)	0.7	0.8	0.8	0.6	0.6	0.9
μ (day-1)	0.4	0.3	1.3	0.4	0.5	0.8
qP ($pg \cdot c^{-1} \cdot d^{-1}$)	18	19	0.9	16	14	1.2
CSPR ($pL \cdot c^{-1} \cdot d^{-1}$)	22	21	1.0	22	22	1.0
Glucose ($g \cdot L^{-1}$)	8.6	6.8	1.3	7.6	6.0	1.3
Lac ($g \cdot L^{-1}$)	2.3	1.4	1.6	2.1	1.5	1.4
Glu ($mg \cdot L^{-1}$)	154	30	5.2	284	105	2.7
Gln ($mg \cdot L^{-1}$)	27	88	0.3	17	0	n.a.
NH4+ ($mg \cdot L^{-1}$)	53	34	1.6	83	27	3.0
qGluc cons. ($pg \cdot c^{-1} \cdot d^{-1}$)	213	187	1.1	244	224	1.1
qLac prod. ($pg \cdot c^{-1} \cdot d^{-1}$)	63	26	2.4	59	31	1.9
qGlu cons. ($pg \cdot c^{-1} \cdot d^{-1}$)	9.1	7.7	1.2	10.3	12.0	0.9
qGln prod. ($pg \cdot c^{-1} \cdot d^{-1}$)	0.7	1.7	0.4	0.5	−0.3	−1.6
qNH4+ ($pg \cdot c^{-1} \cdot d^{-1}$)	−0.6	−0.9	0.6	0.7	0.2	3.0
qLac/qGlu ($mol \cdot mol^{-1}$)	0.6	0.3	2.1	0.5	0.3	1.7
Residual Osm ($mOsm \cdot kg^{-1}$)	326	330	1.0	297	283	1.1

Glucose concentration and specific consumption matched the bioreactor behavior. However, other small-scale metabolite profiles showed higher deviation from the bioreactor behavior because of the semi-continuous perfusion method (where spent medium is completely changed once a day), which contrasts with a truly continuous dilution with fresh medium in perfusion bioreactors.

Residual lactate, glutamate, and ammonia concentrations were higher in SDM cultures. One explanation for higher lactate levels is the higher starting pH in SDM. In non-instrumented shake tubes, pH and DO was not measured or controlled and cells received fresh medium with a rather high pH. Only after a certain incubation time was the culture pH slowly reduced by the fixed CO2 concentration in the incubator and by the accumulation of secreted lactate. Various reports indicate that specific lactate secretion is enhanced at higher pH. Furthermore, no metabolic shift was induced that would otherwise lead to consumption of the produced lactate.

Differences in metabolite behavior can be attributed to the semi-continuous perfusion regimen applied in small-scale models and may be improved by partly exchanging the medium twice a day to achieve a more continuous perfusion regimen, as seen in true perfusion bioreactor cultures.

CONCLUSIONS

In this study, small-scale models based on non-instrumented shake tubes were optimized and applied to perfusion runs using

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two different high-performing perfusion media. Optimal parameters for semi-continuous perfusion scale down models were evaluated and defined, including culture vessel (50 mL shake tubes), shake angle (90°), shake speed (220 rpm), shake amplitude (50 mm), and working volume (10 mL). More than 20 parallel cultures can be operated by only one (trained) operator and this number can be expanded using robotic systems. Thus, the optimized perfusion scale-down models are suitable for DoE and MVDA-assisted high-throughput screening and for optimization in continuous and intensified bioprocess applications. The models proved to be highly suitable to systematically investigate various daily bleeding rates to enhance culture performance and to define optimal CSPR setpoints for larger perfusion bioreactor runs.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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