Biochemical Engineering Journal 195 (2023) 108906

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Development of mL-scale pseudo-perfusion methodologies for high-throughput early phase development studies

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ARTICLE INFO

Keywords:
CHO cells
Microscale
Perfusion culture
Monoclonal antibodies
High throughput

ABSTRACT

Small-scale devices enable rapid development and optimization, and while many exist for fed-batch cultures, there are few comparable devices for high cell density perfusion cultures. To address this gap, pseudo-perfusion methodologies were developed in microwell plates at mL-scale capable of achieving many of the specific characteristics of perfusion culture, including high cell density, cell retention and increased productivity. Pseudo-perfusion was achieved via sedimentation or centrifugation of 24 microwell plates prior to media exchange 1–2 times daily, generating separation efficiencies higher than 90%. Media exchanges commenced on day 3 and achieved perfusion rates of 0.5–1.8 vessel volumes per day (VVD). Pseudo-perfusion methodologies resulted in maximum viable cell densities (VCDs) of up to 42 × 10^6 cells mL^{-1}, 4.2-fold greater than fed-batch cultures. Volumetric productivities increased by 1.9-fold, generating industrially relevant productivities. Similar performance was observed between sedimentation and centrifugation methodologies, with minor deviations attributed to elongated manipulation times and lower packed cell density prior to separation when sedimentation was used. The microwell plate (MWP) experiments were validated at the 5 L scale and showed comparability in metabolite and growth profiles. The work presents the development of a robust tool for high-throughput development studies which is sensitive to changes in media composition and exchange rate, which could be reliably used for initial screening of high cell density perfusion cultures.

1. Introduction

As the production of therapeutic proteins, such as monoclonal antibodies (mAbs), are being produced at increasingly larger scales, the industry is moving towards continuous processing to achieve increased efficiency [1]. The implementation of perfusion culture methods offers greater productivity, a smaller facility footprint and reduced cost of goods [2] but widespread uptake of the technology has been hindered by high risk of batch failure and by the complexity of set-up. Recent technological advancements have led to reduced failure rates, increased the productivity of perfusion cultures and reduced batch to batch variability when compared to fed-batch [3,4]. Additionally, the long-term steady-state perfusion culture operation ensures consistent product quality [5], which has led to regulatory bodies to drive initiatives to promote the implementation of continuous biomanufacturing [6].

It has been suggested that the success of perfusion culture methodologies relies on the ability to reduce perfusion rate, ideally to rates below 1 vessel volume per day (VVD) [7]. This ensures the achievement of high titers using minimal liquid throughput, thus reducing the operating costs. In order to achieve a reduction in perfusion rate, cell lines must be carefully selected, and media optimized to ensure appropriate nutritional depth and osmolality. The use of high-throughput mL-scale devices offer the possibility to select the best-performing cell lines and conduct media studies by running experiments in parallel, thus reducing timescales and costs associated with early phase development. While devices like microwell plates and micro-bioreactors have been widely used for fed-batch cultures, equivalent platforms for perfusion culture are still in development. As perfusion operation requires optimisation of multiple process parameters, this can be a resource-intensive and low-throughput when performed in L-scale benchtop bioreactors. As an alternative, pseudo perfusion in shake flasks, spin tubes or microwell plates is often used.

A few examples of these pseudo-perfusion systems have previously been described, with discrete media exchange and cell retention

Abbreviations: **CHO,**, Chinese hamster ovary; **CSPR**, Cell specific perfusion rate; **mAbs,** Viable cell density, Monoclonal antibodies **MWP** Microwell plate **VCD**; **VVD**, Vessel volumes per day.

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https://doi.org/10.1016/j.bej.2023.108906
Received 29 January 2023; Received in revised form 15 March 2023; Accepted 22 March 2023
Available online 23 March 2023

incorporated into pre-existing fed-batch processes for CHO cell culture. Examples include shake flasks, spin tubes [7–13] and mL-scale bioreactors [14–17]. Pseudo perfusion shake flasks have achieved cell densities of 70 × 10^6 cells mL^{-1} [18]. However, shake flasks are labor intensive and their operation difficult to automate, while this is highly desirable when screening 100's of conditions. Spin tubes usually have working volumes between 10 and 50 mL and are shaken by a speed N between 220 and 320 rpm. Cell separation is achieved via centrifugation prior to media exchange, varying between 40% and 100% of the working volume. Villiger-Oberbek et al. reported viable cell densities up to 50 × 10^6 cells mL^{-1} [19] while Karst et al. maintained a stationary phase culture at 15 × 10^6 cells mL^{-1} to perform dynamic metabolic analysis [20]. The utilisation of ambr15® to mimic perfusion cultures have been reported, either utilising sedimentation as a cell retention mechanism [14,15] or a chemostat mode [21]. The sedimentation methodology describes a period of 40–60 min to allow cell settling, which includes the temporary shutdown of pH, DO and stirrer controls before supernatant removal and pulse media additions performed up to 8 times daily. However, the extended period of control shutdown during sedimentation might cause pH and DO spikes that become detrimental to cell health as the culture progresses. Chemostat modes include pulse media additions, without any cell retention step, whilst maintaining a constant volume. Viable cell densities of up to 20 × 10^5 cells mL^{-1} have been reported [21], and whilst the periodic media exchange can be considered a stepping stone towards mimicking the large volumes of media exchange in perfusion methodologies, this methodology is limited by the absence of a cell retention step. Additionally, the ambr250® can be used to mimic perfusion culture, however this might involve significant purchase and operation costs [17]. All methods presented so far have working volumes between 10 and 250 mL, however, to conduct initial screening and optimization studies, a platform with lower working volumes would allow for high throughput experimentation at reduced cost.

Microwell plates have often been utilised for high throughput development studies of batch and fed-batch cell cultures [22–25]. These have the potential to achieve a significant reduction in operational costs and vastly increase experimental throughput in comparison to shake flasks, spin tubes and microbioreactors [26]. The use of plate covers minimises evaporation whilst maximising oxygen transfer rates, meaning that microwell cultures are able to be sustained in a sterile environment for long times [27]. Microwell plates rely on headspace oxygen transfer, with shaking speed and surface area to volume ratio determined as important influencing factors to maximise oxygen transfer and therefore the gas-liquid mass transfer coefficient k_La [22,27,28]. While k_La increases with increased shaking frequency, a critical shaking speed might be reached at which detrimental effects to cell culture are observed [22]. It was reported that k_La of 5–55 hr^{-1} support up to 10^8 cells mL^{-1} [28], with a k_La of 12 hr^{-1} reported for 24 round well plates with a 1 mL fill volume shaken at 225 rpm and covered with a gas-permeable membrane [22]. Published k_La values in microwell plates therefore suggest oxygen should not be the limiting factor for CHO cell growth at cell densities higher than 10^7 [26].

Huang et al. developed a methodology in 24-deep-well plates with a working volume of 3 mL in which cells are seeded at the target cell density, between 20 and 30 × 10^6 cells mL^{-1}. In their work constant cell density is maintained via daily centrifugation and media exchange using 100% of the working volume [29]. The study of stationary phase cultures showed to be a useful tool for metabolic profiling, however the analysis of the stationary phase only limited the ability to select best-performing cell clones and media. In this work the development of a pseudo-perfusion methodology in microwell plates capable of achieving the characteristics of bioreactor perfusion culture, namely cell retention capabilities, high cell densities and increased productivity, is described. Two key cell retention methodologies, sedimentation and centrifugation, have been developed in microwell plate format and integrated with the culture step to allow for comparison and critical evaluation of each method as competing recovery option.

2. Materials and methods

2.1. Cell culture and media

An industrial IgG producing GS-CHO K1 cell line (Lonza) was used in all experiments. Cells were maintained in CD-CHO medium supplemented with 25 μM methyl sulphoximine (MSX) and passaged twice per week. The cells were cultivated in non-baffled 250 mL shake flasks with vented caps (Corning) with working volume of 50 mL and a seeding density of 0.3 x10^6 cells mL. Cultures were placed in a CO_2 incubator (MCO-19AIC; Sanyo) at 37 °C, with 5% CO_2 and were agitated on shaker tables with an orbital diameter of 25 mm (CO2 Resistant Shaker; Thermo Fisher Scientific). EfficientFeed™ B (Gibco®), a liquid nutrient supplement, was utilized for feeding in fed-batch cultures and to supplement CD-CHO in pseudo-perfusion cultures. All microwell cultures were conducted in 24 standard round well ultra low attachment plates (Corning) covered with a Duetz sandwich lid and a metal clamp (Enzyscreen).

2.2. Microwell culture

GS-CHO cells were sub-cultured in 250 mL vented shake flasks at alternating intervals of 3 or 4 days at a seeding density of 3 × 10^5 viable cells mL^{-1} prior to seeding into microwell plates. Fed-batch and pseudo-perfusion cultures were seeded at 2 × 10^6 viable cells mL^{-1} with a working volume of 1.2 mL. All cultures were incubated at 37 ℃ and shaken at 220 rpm with an orbital diameter of 25 mm. Relative humidity was controlled and CO_2 maintained at 5%. For fed-batch cultures, feeding commenced on day 3 for a period of 5 consecutive days. Feeding conducted as described by Silk and involved a 6% v/v bolus addition of nutrient supplement (EfficientFeed™ B), followed with a 2.5% v/v bolus of 10x diluted bicarbonate solution (0.75 M Na_2CO_3, 0.5 M $NaHCO_3$) to control pH [23]. Evaporation was monitored via daily weight measurements. Control experiments showed no statistically significant deviation in evaporation across the plate (*data not shown*). Due to the limited culture volume, sampling was achieved via a sacrificial well approach [30] with samples taken on days 3 and 5–9 prior to media exchange. Three separate wells were sampled each day allowing for technical triplicates, where error bars represent the standard deviation (n = 3). Preliminary experiments were conducted to assess reproducibility of cell culture in different plates and to quantify well-to-well variability within one plate. A maximum variation of 10% was obtained for cell counts and viability (n = 3, *data not shown*).

2.3. Pseudo-perfusion culture

Media exchanges were started on day 3 and continued until the termination of the culture. A cell retention step was developed using either (1) sedimentation or (2) centrifugation techniques. When sedimentation was used, plates were removed from the shaker and allowed to settle for 28 min at 37 ℃ and 5% CO_2. Alternatively, plates were centrifuged in a benchtop microplate centrifuge at 60g for 5 min. A defined volume of supernatant was then removed and fresh media added. The volume of fresh media was calculated as the volume of supernatant removed plus an additional volume x to account for a total daily evaporation of 3%. The volume x equals 36 μL (3%) and 18 μL (1.5%) for medium exchanges once or twice per day, respectively. The media exchanged was CD-CHO media supplemented with EfficientFeed™ B at 5, 15, 30 or 45% v/v.

Three and four perfusion rates were evaluated for both the sedimentation and centrifugation options, respectively. This was achieved by varying the volume of exchanged media from 600 to 1100 μL and by repeating exchanges up to twice daily to achieve relative media exchange rates between 0.5 and 1.8 VVD.

2.4. 5 L Bioreactor operation

A 5 L stirred bioreactor (Biostat B-DCU; Sartorius, Epsom, UK) was operated in perfusion mode for 14 days at 37℃, a DO setpoint of 30% and a pH of 7.2. Temperature was controlled using a heating jacket, and pH was controlled using base and CO_2 additions. DO was maintained by sparging oxygen and air, and by increasing the impeller speed from 260 to 300 rpm over two days. A horseshoe sparger was used, and agitation was provided by a three blade pitched impeller with a 45° pitch. Cells were seeded at 2×10^6 cells mL^{-1} and cultured in batch mode until perfusion was initiated on day 3. Cell retention was achieved using a tangential flow filtration (TFF) mPES hollow fiber, with an internal diameter of 1 cm, filter area of 480 cm^2 and pore size of 0.2 μm (Spectrum MiniKros, Repligen). The recirculation flowrate of 400 mL min^{-1} produced a shear rate of 1000 s^{-1} using a PuraLev i30SU pump (Levitronix). A perfusion flowrate of 1 VVD was maintained and foaming was controlled using daily additions of 20 mL of 15% antifoam (Antifoam C emulsion; Sigma). The media composition used was CD-CHO blended with 15% Efficient Feed B (ThermoFisher).

2.5. Analytical methods

Viable cell concentrations, viabilities and diameters were determined using a ViCell-XR (Beckman Coulter), where necessary samples were diluted with phosphate buffered saline (PBS). Remaining culture volume was centrifuged and the supernatant frozen for subsequent analysis of metabolites on a bioprofile flex (NOVA Biomedical) and IgG titer by Protein G HPLC (GE Healthcare). Cumulative cell density (IVCD), specific glucose consumption (q_{gluc}), specific lactate consumption (q_{Lac}) and specific productivity (q_{Ab}) were calculated following Villiger-Oberbek et al. [19] and according to Eq. 1:

$$IVCD_n = IVCD_{n-1} + \left(\left(\frac{VCD_n + VCD_{n-1}}{2} \right) \times (t_n - t_{n-1}) \right) \quad (1)$$

Where $IVCD_n$ and $IVCD_{n-1}$ are integrated viable cell densities calculated for time t_n and t_{n-1} respectively. The specific glucose consumption was determined using Eq. 2:

$$q_{gluc} = \frac{\left(c_{gluc,n-1} \times (1 - D_n) + \left(m_{gluc} \times D_n\right)\right) - c_{gluc,n}}{\Delta IVCD_n} \quad (2)$$

Where $c_{gluc,n}$and $c_{gluc,n-1}$ are glucose concentrations measured in the media at time t_n and t_{n-1} respectively; D_n is the daily dilution at time t_n and m_{gluc} is the concentration of glucose in exchanged media. m_{gluc} is 7, 9, 12 and 15 g L^{-1} (39, 50, 67 and 83 mmol L^{-1}) for 5%, 15%, 30% and 45% media blends respectively. The cell specific lactate and mAb productivity was determined using Eq. 3:

$$q_x = \frac{\left(p_{x,n} - \left(p_{x,n-1} \times (1 - D_n)\right)\right)}{\Delta IVCD_n} \quad (3)$$

Where x is the product (Ab) or lactate (Lac) and $p_{x,n}$ and $p_{x,n-1}$ are product or lactate concentration. Cell specific perfusion rate (CSPR) was calculated following Ozturk [16] according to Eq. 4:

$$CSPR = \frac{D_n}{VCD_n} \quad (4)$$

Volumetric productivity, VP, was calculated according to Eq. 5:

$$VP = \frac{\sum_{t=o}^{t=n} p_{Ab} \times V_s}{V \times t_n} \quad (5)$$

Where V_s is the volume of product containing supernatant and V is the volume of cell culture.

3. Results

3.1. Development of pseudo-perfusion cultures in MWP's

A fed-batch CHO culture was initially established in 24 microwell plate format at a volume of 1.2 mL with associated analytics. Following the establishment of fed-batch protocols, sedimentation and centrifugation methodologies were incorporated into microwell plate cultures for pseudo-perfusion studies. This initially involved media exchange with CD-CHO at 1 VVD and produced higher cell densities in comparison to fed-batch mode, as expected. Following establishment of pseudo-perfusion protocols, exchange media was studied to optimize performance. Fig. 1A and B show viable cell counts and viability, respectively, for optimized fed-batch and pseudo-perfusion cultures at different media concentrations. The media optimization study was performed using CD-CHO supplemented with 5, 15 30% and 45% v/v Efficient-Feed™ B at a perfusion rate of 1 VVD. In the fed-batch culture the maximum VCD was 10.6×10^6 cells mL^{-1} and viability started dropping after 7 days of cultivation. Pseudo-perfusion techniques generate VCDs significantly greater than fed-batch and achieve comparable performance for all but the lowest and highest media supplementation. In sedimentation cultures, all media blends showed comparable media performance until day 6, with deviations observed on day 7. The use of 45% media resulted in a significant decline in VCD and viability, from 96% to 59%, on day 7. The sudden viability decrease observed coincided with a consistently increased supplementation of glucose, which reached a maximum of 13 g L^{-1} (72 mmol L^{-1}) (*data not shown*) in the culture on day 6 prior to media exchange. Further, a lactate spike was observed on day 7–3 g L^{-1} (33 mmol L^{-1})(*data not shown*) with corresponding high specific lactate productivities (q_{Lac}) coinciding with a reduction of pH to values below 6.5 In this work glucose depletion limited growth for 5% media blend cultures while for cultures with 30% Feed B cell growth was limited by high glucose concentrations of 8 g L^{-1} (44 mmol L^{-1}), and lactate spikes up to 2.1 g L^{-1} (23 mmol L^{-1}) on day 7 (data not shown). The best performing media blend was 15%, which reached a maximum VCD of 21.6×10^6 cells mL^{-1} on day 7 and maintained a viability of higher than 96%. Glucose concentration was well controlled between 1.2 and 4.5 g L^{-1} (7–25 mmol L^{-1}) throughout the culture duration. A 2-fold increase in VCD was achieved with this media blend in comparison to fed-batch mode, which generated a maximum VCD of 10.8×10^6 cells mL^{-1} on day 7. Growth curves for centrifugation cultures are similar with the exception of the highest and lowest media blends. Similarly, sedimentation cultures, with a 15% media blend resulted in the highest cell density, 23.4×10^6 cells mL^{-1} and maintained a viability higher than 96%. Glucose concentration remained well between 1 and 3.6 g L^{-1} (6–20 mmol L^{-1}) between days 1–6, however near-depletion was observed with concentrations of 0.12 g L^{-1} (0.67 mmol L^{-1}) on day 7. Separation efficiencies for sedimentation cultures were found to be between 90% and 99%, but the delicate cell pellet formed in this case meant that separation efficiencies were less consistent than centrifugation cultures, which were between 93% and 98% (Fig. 1C & D). Fig. 2 compares volumetric productivity for fed-batch and pseudo-perfusion cultures at different media concentrations. Although such comparison is challenging, the volumetric productivity of the most productive days of perfusion and fed-batch can be compared to assess performance [31] and such method was used in this work to compare fed-batch and pseudo perfusion. All media blends produced up to 2-fold greater mAb per liter of culture per day compared to fed-batch. Centrifugation cultures had slightly improved performance than the corresponding sedimentation culture, with 15% blend media producing the highest volumetric productivity across perfusion modes. It is clear that productivity was not directly reliant on VCD with worse performing cultures, such as 5% media centrifugation cultures, producing some of the greatest volumetric productivity.

Overall, comparable performance was observed between sedimentation and centrifugation cultures. Promising increases in VCD

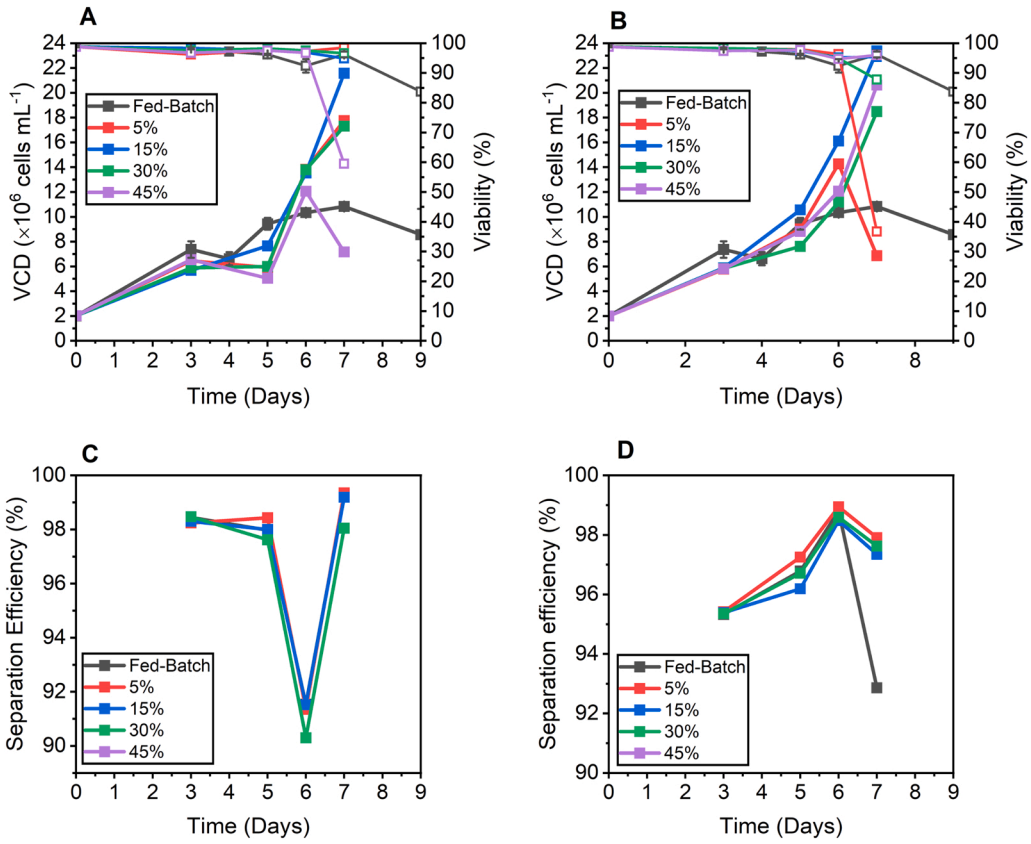

Fig. 1. Performance comparison of fed-batch, sedimentation and centrifugation cultures exchanged with media supplemented with 5%, 15%, 30% and 45% EfficientFeed™ B v/v. at 1 VVD (A) VCD (solid squares) and viability profiles (empty squares) of fed-batch and sedimentation cultures. (B) VCD (solid squares) and viability profiles (empty squares) of fed-batch and centrifugation cultures. (C) Separation efficiency of sedimentation cultures. (D) Separation efficiency of centrifugation cultures. Error bars represent the standard deviation, n = 3.

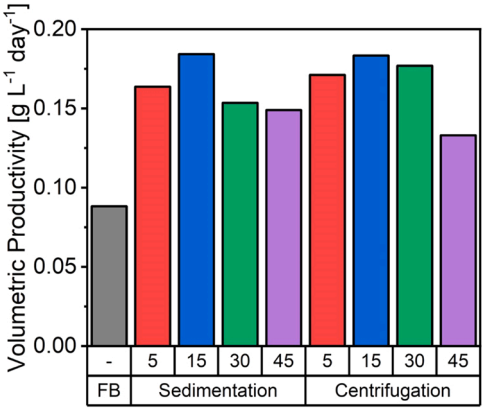

Fig. 2. Comparison of volumetric productivities of fed-batch and sedimentation and centrifugation pseudo-perfusion cultures, exchanged with media supplemented with 5, 15, 30 or 45% EfficientFeed™ B. Values were calculated using cumulative mAb produced across culture duration taken from 6 separate wells due to the sacrificial approach, with final volumetric productivities calculated with Eq. 5.

compared to fed-batch cultures were shown for a range of conditions, alongside consistently high separation efficiencies (higher than 90%) and over 2-fold improvements to volumetric productivity. 15% media represented the best-performance across sedimentation and centrifugation cultures and was therefore selected for use in all subsequent experiments.

3.2. Impact of varying perfusion rates on cell culture performance

In order to assess the robustness of the pseudo-perfusion methodology as a tool for early phase development of perfusion culture, volume and frequency of media exchange were varied to study the impact of perfusion rates on consistency and culture performance. Fig. 3 shows the VCD, viability and separation efficiencies of pseudo-perfusion cultures at varying media exchange rates. To achieve a perfusion rate of 0.5 and 0.75 VVD, one daily media exchange was performed while for 1.5 and 1.8 VVD cultures half the volume was exchanged twice per day. To avoid cell pellet disruption in sedimentation cultures, supernatant removal was capped at a maximum of 75% of total volume, with media exchange performed a maximum of twice daily. Therefore, 1.5 VVD is the maximum perfusion rate than can be tested via sedimentation and 1.8 VVD was run in centrifugation mode only. No significant difference in growth kinetics was observed between sedimentation and centrifugation in the early phase cultures under otherwise identical conditions for all exchange rates analyzed. VCDs for sedimentation cultures on day 7 was between 20.7 and 22.8 $\times$ 10^6 cells mL^{-1}, with viabilities higher than 94.0%. Centrifugation cultures at different perfusion rates were comparable until day 5, with VCD improvements of up to 25% observed from day 6. VCDs on day 7 was between 21.4 and 25.2 $\times$ 10^6 cells mL^{-1}, with viabilities higher than 91.0%. Cultures with a medium exchange of 1.5 VVD were maintained till day 9 to extend the growth period and showing continued improvements in VCD. Low exchange rate, 0.5 and 0.75 VVD, centrifugation cultures resulted in high viabilities at 95.5% and 91.1%, respectively, and achieved maximum VCDs of 33.3 $\times$ 10^6 cells mL^{-1} on day 9. Fig. 3C and D compare sedimentation and centrifugation cultures at 1.5 VVD and showed equivalent maximum VCDs on day 9 of 32.2 and 34.2 $\times$ 10^6 cells mL^{-1} respectively. Increasing the perfusion rate from 0.5 to 1.5 VVD resulted in a minor impact on VCD, however a further increase to 1.8 VVD in centrifugation cultures showed a 23% improvement in VCD, achieving 42.2 $\times$ 10^6 cells mL^{-1} with a viability of 96.1% on day 9.

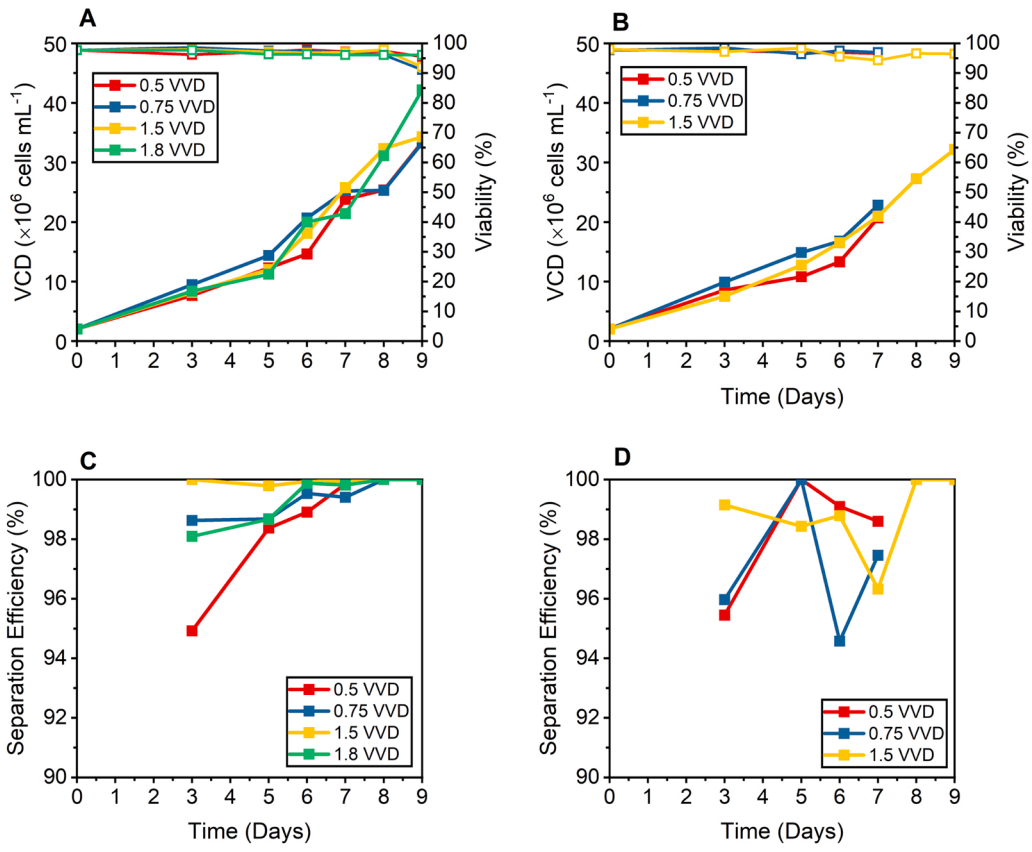

Fig. 3. Performance comparison of sedimentation and centrifugation cultures exchanged with media supplemented with 15% EfficientFeed™ B v/v and exchanged at 0.5, 0.75, 1.5 and 1.8 VVD (A) VCD (solids squares) and viability profiles (empty squares) of sedimentation cultures. (B) VCD (solid squares) and viability profiles (empty squares) of centrifugation cultures. (C) Separation efficiency of sedimentation cultures. (D) Separation efficiency of centrifugation cultures. 1.8 VVD was not tested for sedimentation due to a maximum exchange volume of 75% taken twice daily.

3.2.1. Evaluating performance of pseudo-perfusion and fed-batch cultures

Table 1 shows a summary of performance data, calculated as outlined in Eqs. 1–5. Constant perfusion rate is maintained in the experiments, resulting in a decrease of CSPR as VCD increased with time. The CSPR reported is the minimum reached for each culture condition. For lower perfusion rates, 0.5 and 0.75 VVD, minimum CSPR achieved were comparable between 0.02 and 0.03 nL cell^{-1} day^{-1}. Higher perfusion rates showed a close agreement between sedimentation and centrifugation and 1.5 and 1.8 VVD cultures, obtaining minimum CSPRs between 0.05 and 0.06 nL cell^{-1} day^{-1}. Specific production of antibody (q_{Ab}), closely correlated with perfusion rates for both sedimentation and centrifugation cultures. Low perfusion rates obtained a reduced q_{Ab} compared to fed-batch cultures and are comparable across sedimentation and centrifugation methodologies, between 2 and 2.5 pg cell^{-1} day^{-1} compared to fed-batch at 3.2 pg cell^{-1} day^{-1}. However, higher perfusion rates, 1.5 and 1.8 VVD, increased q_{Ab} by up to 1.9-fold for sedimentation cultures at 1.5 VVD and 1.5-fold for centrifugation cultures at 1.8 VVD (5.9 and 4.9 ×10^{-5} g cell^{-1} day^{-1} respectively). The supernatant titer throughout the culture was reduced in pseudo-perfusion cultures compared to fed-batch, which achieved an end-point titer of 0.8 g L^{-1}. Titers in high perfusion rate cultures were reduced by over 50%, to an average value between 0.28 and 0.35 g L^{-1} throughout the culture.

Table 1
Performance data for fed-batch, sedimentation and centrifugation pseudo-perfusion cultures exchanged with CD CHO media supplemented with 15% EfficientFeed™ B at exchange rates of 0.5, 0.75, 1.5 and 1.8 VVD.

	Fed-Batch	Sedimentation			Centrifugation			
		1.5VVD	0.75VVD	0.5VVD	1.8VVD	1.5VVD	0.75VVD	0.5VVD
VCD day 7 (x10^6 cells mL^{-1})	10.8	25.8	22.8	20.7	21.4	20.9	25.2	23.8
VCD day 9 (x10^6 cells mL^{-1})		32.2	N/A	N/A	42.2	34.3	33.3	33.4
Number of exchanges (day^{-1})	N/A	2	1	1	2	2	1	1
Volume per exchange (mL)	N/A	0.9	0.9	0.6	1.1	0.9	0.9	0.6
CSPR$_{min}$ (nL cell^{-1} day^{-1})	N/A	0.06	0.03	0.03	0.05	0.05	0.02	0.02
[a]q_{Ab} (pg cell^{-1} day^{-1})	3.16	5.87	2.07	2.63	4.89	3.56	2.23	2.45
[a]Supernatant Titer (g L^{-1})	0.88	0.28	0.46	0.58	0.35	0.29	0.61	0.74
Volumetric productivity (g L^{-1} day^{-1})	0.09	0.28	0.20	0.17	0.42	0.29	0.31	0.24
[a]q_{Lac} (x10^{-5} g cell^{-1} day^{-1})	2.47	5.75	1.65	2.21	4.46	4.30	0.62	0.09
[a]q_{Gluc} (x10^{-4} g cell^{-1} day^{-1})	4.38	2.73	2.32	3.13	3.24	2.80	1.67	1.46

N/A not applicable
The perfusion rate of 1.8 VVD was investigated only for the cell retention with centrifugation as the maximum amount of medium exchanged was capped at 75% for cultures with medium exchanges twice a day.
[a] For pseudo-perfusion cultures, values are given as average over the entire cultivation time.

Lower perfusion rates, however, produced titers more comparable to fed-batch cultures due to the decreased dilution rate. This observation was expected due to the fact that product can accumulate in fed-batch cultures while in semi-perfusion cultures product is removed during the medium exchanges. Normalized parameter such as the volumetric and cell-specific productivities (q_{mAb}) allow for comparison between different modes of operation. Whilst the q_{mAbs} were lower between fed-batch and semi-perfusion at low medium exchange rates for both cell retention techniques, at high perfusion rates the q_{mAb} of pseudo-perfusion cultures was increased compared to fed-batch operations (Table 1). Fig. 4 compares the volumetric productivities of pseudo-perfusion cultures at varying media exchange rates. Centrifugation cultures at 1.8 VVD achieved a volumetric productivity of over 40% greater than all other pseudo-perfusion conditions and 4-fold greater than fed-batch of 0.09 g L^{-1} day^{-1} (Table 1). Sedimentation cultures showed a decrease in volumetric productivity with decreased perfusion rate, from 0.28 to 0.17 g L^{-1} day^{-1} for 1.5 and 0.5 VVD, respectively. Centrifugation cultures performed much more consistently, with volumetric productivities in the range of 0.24–0.31 g L^{-1} day^{-1} for 0.5–1.5 VVD cultures. Specific glucose consumption, q_{gluc}, was reduced in pseudo-perfusion cultures compared to fed-batch cultures. Within centrifugation cultures, reducing perfusion rate resulted in reduced q_{gluc}, which coincided with limited availability of glucose in low media exchange rate cultures. High perfusion rate cultures showed an increased specific lactate production, q_{Lac}, compared to fed-batch cultures by up to 2-fold.

3.3. Comparison of pseudo-perfusion MWP to 5 L perfusion bioreactor

Fig. 5 shows the comparison of perfusion bioreactor at the 5 L scale and MWP in pseudo-perfusion using centrifugation for cell retention and a medium exchange rate of 1VVD. Fig. 5 A shows that both methodologies achieved comparable growth till day 8 (the end of MWP cultivation) the VCD's of the MWP and bioreactor were 40 × 10^6 cells mL^{-1} and 48 × 10^6 cells mL^{-1} respectively. However, cell growth continued to VCD of 69 × 10^6 cells mL^{-1} for the bioreactor due to the extended cultivation time. Fig. 5D shows the mAb concentration achieved each day for the two systems, with the MWP achieving a 65% higher maximum concentration of 0.68 g L^{-1} on day 8. For the comparison of two different operation modes, pseudo-perfusion (batch-wise medium exchange) and perfusion (continuous medium exchange), the volumetric and cell-specific productivities were analyzed (Table 2). The volumetric productivities of each system were comparable with the MWP achieving 0.23 and the bioreactor achieving 0.26 g L^{-1}. The q_{mAb} obtained for the 5 L bioreactor were slightly reduced achieving 4.42 pg $cell^{-1}$ day^{-1}, in comparison to the q_{mAb} of 5.45 pg $cell^{-1}$ day^{-1} obtained with the MWP culture in pseudo-perfusion. For the perfusion bioreactor limitations of oxygen were observed after day 4, where a DO of 30% was no longer maintained (data not shown). Fig. 5B shows a comparable glucose concentration for the two systems and Fig. 5 C shows a comparable lactate concentration. However, the bioreactor lactate concentration spiked to over double the MWP on the third day before once again reducing to comparable levels.

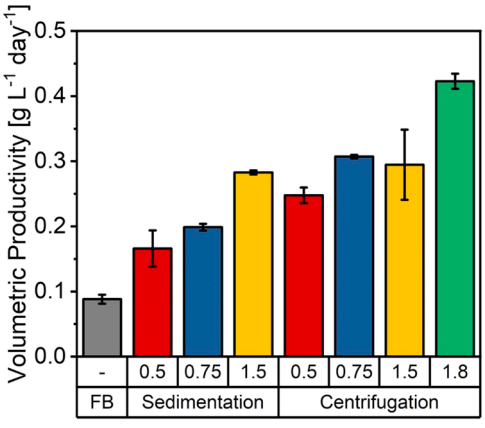

Fig. 4. Comparison of volumetric productivities of sedimentation and centrifugation pseudo-perfusion cultures, exchanged with media supplemented with 15% EfficientFeed™ B at exchange rates of 0.5, 0.75, 1.5 and 1.8 VVD. The values represent the average of volumetric productivities over the cultivation duration. The perfusion rate of 1.8 VVD was not tested for sedimentation due to a maximum exchange volume of 75% taken twice daily. Error bars represent the standard deviation (n = 2) for pseudo perfusion and (n = 3) for fed-batch.

4. Discussion

This study demonstrated the successful implementation of different cell retention techniques into the microwell plate platform. The ability to retain cells (either by centrifugation or sedimentation) and the regular medium exchanges allowed to achieve HCD at a range of perfusion rates from 0.5 to 1.8 VVD, representing up to 4-fold improvements compared to fed-batch processes. An initial screening for medium composition showed different culture performance, which makes the system promising for the use in early phase media screening applications. Volumetric productivity is increased in pseudo-perfusion cultures by up to 2–4 fold for low and high VVD respectively, and specific antibody production is increased over 1.5 fold for high perfusion rate cultures.

4.1. Analysis of pseudo-perfusion methodologies for media screening applications

The purpose of the media screen was (1) to select the most appropriate media for subsequent studies, ensuring that achieving the full system capability was not limited by lack of adequate nutrients and (2) to demonstrate the system capabilities as a screening tool. Media selection is a vital phase in perfusion culture development, with several selection parameters: cost, consumption, nutritional depth and osmolality [32]. Media must have the appropriate nutritional depth, defined as the number of cells supported per mL, without increasing osmolality such that it becomes damaging to cell growth and viability. An osmolality higher than 450 mOsm kg^{-1} is considered to become damaging to cell growth and viability [32,33]. The osmolality of all media studied are between 320 and 355 mOsmol kg^{-1}, and therefore detrimental osmotic effects are not considered in this instance. In the context of optimization of bioreactors at 10–100's L-scale, cost and consumption rate could be incorporated as selection parameters to screen for media that provides the best cost to benefit ratio. Pseudo-perfusion methodologies were found to be sensitive to changes in media composition, promising for potential use in early-phase media screening. In the study, consistent over feeding of glucose lead to spikes in the lactate concentration to 3 g L^{-1} (33 mmol L^{-1}) in sedimentation cultures with 45% feed B. High lactate concentrations (>2 g L^{-1} (22 mmol L^{-1})) were previously shown to cause acidification with a reduction of pH to less than 6.5 in fed-batch cultures [34]. It is possible that the increase of lactate concentration in this culture led to a reduction of pH causing the observed reduction of cell viability. However, due to the small volume of the MWP the monitoring of pH was not possible. The results of media optimization experiments also highlighted an important system limitation. With infrequent media exchanges concentration of nutrients, such as glucose, were not kept constant and toxic byproducts, such as lactate, accumulated for 12–24 hrs before exchange. The result was periodically changing glucose and lactate concentration between media exchanges, as reported in alternative pseudo-perfusion methodologies [7–14]. While this limitation could be mitigated by increasing the number of media exchanges to achieve the desired perfusion rate, a large number of plate manipulations per day could become detrimental to culture performance, increases the risk of contamination and, without automation,

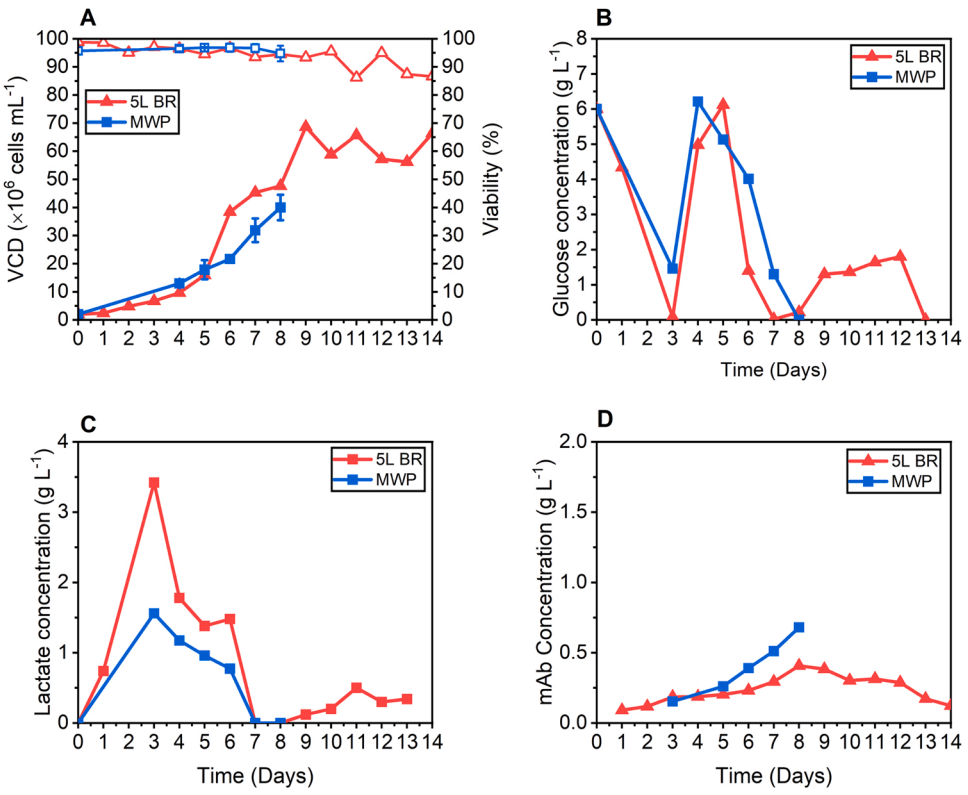

Fig. 5. Comparison of MWP pseudo-perfusion methodology to a 5 L perfusion bioreactor (A) VCD growth curves (solid squares) and associated cell viability (empty squares) (B) Glucose concentration (C) Lactate concentration and (D) mAb concentration. MWP cultures were inoculated at 2×10^6 cells mL^{-1} and operated for 8 days due to the sacrificial well methodology. The medium was exchanged manually with a perfusion rate equal to 1 VVD. The bioreactor was inoculated at 2×10^6 cells mL^{-1} and operated at a perfusion rate of 1 VVD.

Table 2
Performance data of CHO cells in 5 L perfusion bioreactor and pseudo-perfusion with a perfusion rate of 1 VVD. Cells were seeded at 2 × 106 cells mL^{-1} and cultured in CD CHO medium supplemented with 15% EfficientFeed™ B.

	MWP	5 L BR
max. VCD (x10^6 cells mL^{-1})	40	48# 69##
q_{mAb} (pg $cell^{-1}$ day^{-1})*	5.45	4.42
Supernatant Titer (g L^{-1})	0.68	0.40# 0.12##
Volumetric productivity (g L^{-1} day^{-1})*	0.23	0.26

day 8
##day 14
*given as average over the entire cultivation time of 8 and 14 days for MWP and BR, respectively.
VCD: viable cell density, MWP: Microwell plate; BR: bioreactor; q_{mAb}: cell specific productivity; STY: space time yield
Figure legends

reduces the number of plates able to be feasibly managed by one technician, reducing throughput.

4.2. Comparing performance of sedimentation and centrifugation methodologies

Sedimentation and centrifugation produced similar growth curves under otherwise identical conditions in the early phases of culture, with deviation noted from day 6 being within the 10% deviation measured with control experiments. Centrifugation cultures at the highest exchange rate of 1.8 VVD achieved maximum VCDs up to 30% greater than for the lower rates of sedimentation and centrifugation cultures. The key deviation between sedimentation and centrifugation in 1 VVD cultures was thought to be caused by the variation in feeding strategies. Centrifugation techniques resulted in a much smaller packed cell pellet prior to media exchange. Due to the smaller packed cell pellet obtained using centrifugation techniques, the maximum percentage of working volume removed per exchange is greater than in sedimentation cultures. Therefore, to achieve an exchange rate of 1 VVD, sedimentation cultures were exchanged twice, while centrifugation cultures require one exchange, resulting in varying dilution of fresh to spent media. Dilution ratios govern the consistency of the cellular environment, less frequent media exchanges resulted in a less consistent environment and therefore variations in cell culture performance were observed. All subsequent pseudo-perfusion rate experiments presented in this study had matching numbers of daily exchanges in order to match dilution ratios and to enable fair comparison between methodologies.

The separation of sedimentation and centrifugation cultures was reliant on the small density difference between cells and the culture media, less than 100 kg m^{-3}, which in turn led to low gravitational settling velocities 1–15 cm hr^{-1} [35]. Centrifugation cultures enhanced the separation efficiency by increasing the velocity in the gravitational field, which according to Stokes' law increased the settling velocity. The complete media exchange for sedimentation cultures took over 3 times as long as centrifugation, leaving cells more liable to hypoxia during the cell retention step. This increase in manipulation time was believed to influence the culture performance, causing consistent poorer performance of sedimentation cultures compared to centrifugation cultures. The low rpm utilized in centrifugation cultures meant that minimal shear was exerted into the culture [36], meaning that the effects of increased manipulation time in sedimentation cultures was more detrimental to cell health. In terms of separation performance, centrifugation cultures maintained consistent concentration of cells in supernatant throughout a range of exchange rates, media compositions and volumes, meaning separation efficiencies increased with VCD increases, between 92% and 96%. The separation efficiencies of sedimentation cultures however were inversely related to VCD, with increases in VCD causing a larger volume of settled cells thus poorer separation. This was particularly prominent for cultures with the greatest exchange volume per exchange, 0.75 and 1.5 VVD. Additionally, the delicate pellet of separated cells was more easily disrupted during manual supernatant removal, which resulted in separation

efficiencies to be more inconsistent, however was always maintained higher than 90%.

4.3. Performance analysis of pseudo-perfusion methodologies

Prolonged pseudo-perfusion cultures to day 9 showed sedimentation and centrifugation techniques were able to produce comparable VCDs to those in previously described alternatives. The maximum VCD of 42.2×10^6 cells mL^{-1} described in this study in centrifugation cultures at 1.8 VVD showed similar performance to Villiger-Oberbek et al., who reported peak VCD of 50×10^6 cells mL^{-1} for best-performing media and clone in spin tubes. Additionally, VCDs of 35×10^6 cells mL^{-1} in deep-well plates and 60×10^6 cells mL^{-1} in spin tubes [11], and 90×10^6 cells mL^{-1} in shake tubes [8]. Culture performance in microwell cultures did not appear to be worse compared to alternative pseudo-perfusion methodologies, despite representing over an 8-fold decrease in volume.

The maintenance of CSPR is commonly implemented in scale-up meaning it is important for CSPR to be measured and to a certain extent controlled in smaL-scale high throughput devices. CSPRs were reported to be in the range of 0.05–0.5 nL $cell^{-1}$ day^{-1}, with lowest CSPRs considered unfeasible for the support of high cell density cultures 40–80 $\times 10^6$ cells mL^{-1} [37]. The low CSPRs between 0.02 and 0.03 g L^{-1} in low VVD cultures could therefore be a limitation for cell growth where VCDs up to 33×10^6 cells mL^{-1} were reached for 0.75 and 0.5 VVD cultures.

Fed-batch-like titers were demonstrated by low VVD cultures. At the lowest exchange rate, 0.5 VVD, where average supernatant titers produced in sedimentation and centrifugation cultures were 0.58 and 0.74 g L^{-1} obtained, comparable to fed-batch processes producing titers of 0.88 g L^{-1}. As expected, an increased perfusion rate and doubling in the number of exchanges for 1.5 and 1.8 VVD cultures resulted in a reduction of daily measured titer by ~50%. The reduction in titer for high VVD cultures correlates to the increased number of exchanges to twice daily, which most likely led to a less distinct accumulation of product in the culture volume. Nonetheless, the volumetric productivities between otherwise identical cultures at varying VVDs were comparable indicating similar culture performance. This is supported when comparing differing VVDs with the same number of daily exchanges showed, as expected, an increased volumetric productivity relating to the increase in product-containing supernatant harvested. Sedimentation cultures exchanged once daily had a lower titer and volumetric productivity compared to corresponding centrifugation cultures, similar q_{mAb} suggested this deviation was due to decreased VCD in sedimentation cultures. pH was monitored daily for MWP cultures and fell to as low as 6.5 when media was only exchanged every 24 hrs. The limited control of pH was a large limitation of the system and, moving forward, integration of monitoring and control of pH as well as DO would be a meaningful addition to improve performance and allow a more realistic comparison to larger scales, controlled culture.

4.4. MWP pseudo perfusion methodology as a tool to predict perfusion bioreactor operation

The comparison of MWP to a 5 L bioreactor regarding growth, productivities and metabolite dynamics showed, comparable dynamics as well as variations, which must be understood to utilize the methodology as a high throughput, scale down methodology for the optimization of perfusion cultures of CHO cells. The bioreactor can achieve a larger maximum VCD, which is most likely attributed to the longer cultivation duration which is supported by the fact the VCDs were comparable until day 8 (end of the MWP cultivation). Nonetheless, the bioreactor's ability to control oxygen levels due to feeding oxygen directly into the culture might contribute to increased VCD when operating for longer periods of time. However, it should be noted the K_La of the two methods are comparable with the MWP achieving 7.4 hrs^{-1} and the bioreactor achieving 6.2–8 hrs^{-1} as the rpm was increased from 260 to 300. The maximum MWP VCD could be used as an appropriate initial set point for CSPR based steady state bioreactor operation. Lactate concentration in the bioreactor spiked to higher levels than the MWP after 3 days of batch mode operation. This is in line with literature, that states higher lactate production is common in large volumes [37]. Despite the differences in the dynamic of lactate concentration, glucose concentrations were comparable between both systems. The analysis of the daily measured titres showed an increase of maximum titer in the MWP, which also occurred when comparing spin tubes to bioreactors [38]. The most likely explanation for this observation is the difference in medium exchange, where for the bioreactor operation the medium was exchanged continuously, for the MWP the medium exchange happened at distinct time points leading to an accumulation of product between two time points. This is further supported by literature, where spin tubes were operated with a similar perfusion rate showing elevated daily measured titres in comparison to a perfusion bioreactor [38]. Furthermore, the comparability of volumetric and cell-specific productivities both calculated over the entire cultivation duration (8 and 14 days) support the claim of comparability between both systems. Despite some differences observed, the MWP methodology can be used as a cheap, high throughput optimisation tool for perfusion operation in bioreactors for the selection of top performing media blends and perfusion rates.

5. Conclusion

A pseudo-perfusion methodology was developed in microwell plates incorporating cell retention capabilities and producing elevated cell densities and volumetric productivities compared to fed-batch cultures. Sedimentation and centrifugation methodologies had similar culture performance, with minor deviations attributed to elongated manipulation times and lower packed cell density prior to separation in sedimentation cultures. Pseudo-perfusion methodologies were shown to be sensitive to changes in media composition, making the system promising for use in early phase development of perfusion cultures. Maximum VCDs achieved in cultures extended until day 9 utilizing pseudo-perfusion methodologies were 3.3–4.2-fold greater than for fed-batch operations for all conditions analyzed. Higher perfusion rates of 1.5 and 1.8 VVD with higher CSPRs, between 0.05 and 0.06 nL $cell^{-1}$ day^{-1}, generated volumetric productivities up to 1.9-fold greater than for fed-batch and elevated q_{Ab}. Pseudo-perfusion methodologies in microwell plates were capable of mimicking many of the specific characteristics of perfusion cultures, with an 8-fold volume reduction compared to previously reported pseudo-perfusion techniques. A chosen set of process parameters (i.e. 1VVD) was transferred in a 5 L perfusion bioreactor showing comparable results for growth and productivity. Pseudo-perfusion methodologies in microwell plates are a robust, high throughput tool for the early phase development and screening of perfusion cultures. The integration of the system into an automated liquid handling device could further expand capabilities, increasing reliability and consistency.

CRediT authorship contribution statement

Molly Tregidgo: Investigation, Data curation, Methodology, Formal analysis, Writing – original draft, Visualization, Conceptualization. **Ciara Lucas:** Data curation, Methodology, Formal analysis, Writing – review & editing, Visualization, Conceptualization. **Marie Dorn:** Formal analysis, Writing – review & editing, Visualization, Methodology, Conceptualization, **Martina Micheletti**: Conceptualization, Resources, Methodology, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgement

The authors would like to acknowledge the funding and support of the UKRI Engineering and Physical Sciences Research Council (EPSRC) CDT Bioprocess Engineering Leadership Grants EP/S021868/1 and EP/L01520X/1 for Miss Ciara Lucas and Miss Molly Tredidgo, respectively. We would like to thank University College London - Pall Corporation Centre of Excellence and Merck & Co Inc. for financial support of Miss Ciara Lucas and Miss Tredidgo's EngD studentships, respectively. Marie Dorn's studentship was co-funded by the AZ-UCL Centre of Excellence and Engineering and Physical Sciences Research Council (EPSRC), the Centre for Doctoral training [Grant ref: EP/S021868/1]. The authors acknowledge useful scientific discussions with Mark Brower and Nuno Pinto at Merck & Co Inc. which have informed this work.

References

[1] N.K. Tripathi, A. Shrivastava, Recent developments in bioprocessing of recombinant proteins: expression hosts and process development, Front. Bioeng. Biotechnol. (2019), https://doi.org/10.3389/fbioe.2019.00420.
[2] J. Pollock, S.V. Ho, S.S. Farid, Fed-batch and perfusion culture processes: economic, environmental, and operational feasibility under uncertainty, Biotechnol. Bioeng. 110 (2013) 206–219.
[3] J. Walther, et al., Perfusion cell culture decreases process and product heterogeneity in a head-to-head comparison with fed-batch, Biotechnol. J. 14 (2) (2019), https://doi.org/10.1002/biot.201700733.
[4] S. Xu, et al., Bioreactor productivity and media cost comparison for different intensified cell culture processes, Biotechnol. Prog. 33 (4) (2017) 867–878, https://doi.org/10.1002/btpr.2415.
[5] K. Konstantinov, C. Goudar, M. Ng, R. Meneses, J. Thrift, S. Chuppa, C. Matanguihan, J. Michaels, D. Naveh, The "push-to-low" approach for optimization of high-density perfusion cultures of animal, Cells 101 (2006) 75–98.
[6] N. Mihokovic, ECI Integrated Continuous Biomanufacturing III, Cascais, Portugal (2017).
[7] N. Gomez, M. Ambhaikar, L. Zhang, C.J. Huang, H. Barkhordarian, J. Lull, C. Gutierrez, Analysis of Tubespins as a suitable scale-down model of bioreactors for high cell density CHO cell culture, Biotechnol. Prog. 33 (2017) 490–499.
[8] P. Mayrhofer, A. Castan, R. Kunert, Shake tube perfusion cell cultures are suitable tools for the prediction of limiting substrate, CSPR, bleeding strategy, growth and productivity behavior, J. Chem. Technol. Biotechnol. vol. 96 (10) (2021) 2930–2939, https://doi.org/10.1002/jctb.6848.
[9] M. Wolf, M. Morbidelli, "Development of mammalian cell perfusion cultures at lab scale: from orbitally shaken tubes to benchtop bioreactors," in, Methods Mol. Biol. vol. 2095 (2020) 125–140.
[10] P. Mayrhofer, D. Reinhart, A. Castan, R. Kunert, "Rapid development of clone-specific, high-performing perfusion media from established feed supplements,", Biotechnol. Prog. vol. 36 (2) (2020) https://doi.org/10.1002/btpr.2933.
[11] J.M. Bielser, J. Domaradzki, J. Souquet, H. Broly, M. Morbidelli, "Semi-continuous scale-down models for clone and operating parameter screening in perfusion bioreactors,", Biotechnol. Prog. vol. 35 (3) (2019) https://doi.org/10.1002/btpr.2790.
[12] M.K.F. Wolf, A. Müller, J. Souquet, H. Broly, M. Morbidelli, Process design and development of a mammalian cell perfusion culture in shake-tube and benchtop bioreactors, Biotechnol. Bioeng. vol. 116 (8) (2019) 1973–1985, https://doi.org/10.1002/bit.26999.
[13] T. Bissinger, et al., Semi-perfusion cultures of suspension MDCK cells enable high cell concentrations and efficient influenza A virus production, Vaccine vol. 37 (47) (2019) 7003–7010, https://doi.org/10.1016/j.vaccine.2019.04.054.
[14] S. Kreye, R. Stahn, K. Nawrath, V. Goralczyk, B. Zoro, S. Goletz, A novel scale-down mimic of perfusion cell culture using sedimentation in an automated microbioreactor (SAM), Biotechnol. Prog. vol. 35 (5) (2019), https://doi.org/10.1002/btpr.2832.
[15] L. Jin, Z.S. Wang, Y. Cao, R.Q. Sun, H. Zhou, R.Y. Cao, Establishment and optimization of a high-throughput mimic perfusion model in ambr® 15, Biotechnol. Lett. vol. 43 (2) (2021) 423–433, https://doi.org/10.1007/s10529-020-03026-5.
[16] H. Schwarz, et al., Small-scale bioreactor supports high density HEK293 cell perfusion culture for the production of recombinant Erythropoietin, J. Biotechnol. vol. 309 (. 2020) 44–52, https://doi.org/10.1016/j.jbiotec.2019.12.017.
[17] M.A. MacDonald, et al., Perfusion culture of Chinese Hamster Ovary cells for bioprocessing applications, Crit. Rev. Biotechnol. vol. 42 (7) (2022) 1099–1115, https://doi.org/10.1080/07388551.2021.1998821.
[18] S. Janoschek, et al., A protocol to transfer a fed-batch platform process into semi-perfusion mode: The benefit of automated small-scale bioreactors compared to shake flasks as scale-down model, Biotechnol. Prog. 35 (2) (2019), e2757.
[19] A. Villiger-Oberbek, Y. Yang, W. Zhou, J. Yang, Development and application of a high-throughput platform for perfusion-based cell culture processes, J. Biotechnol. 212 (2015) 21–29.
[20] D.J. Karst, F. Steinebach, M. Soos, M. Morbidelli, Process performance and product quality in an integrated continuous antibody production process, Biotechnol. Bioeng. 114 (2017) 298–307.
[21] W. Kelly, S. Veigne, X. Li, Z. Huang, E. Schaefer, Optimizing performance of semi-continuous cell culture in an Ambr15^{TM} microbioreactor using dynamic flux balance modelling, Biotechnol. Prog. 00 (2017) 1–12.
[22] T.A. Barrett, A. Wu, H. Zhang, M.S. Levy, G.J. Lye, Microwell engineering characterization for mammalian cell culture process development, Biotechnol. Bioeng. 105 (2010) 260–275.
[23] N.J. Silk, S. Denby, G. Lewis, M. Kuiper, D. Hatton, R. Field, F. Baganz, G.J. Lye, Fed-batch operation of an industrial cell culture process in shaken microwells, Biotechnol. Lett. 32 (2010) 73–78.
[24] S. Markert, K. Joeris, Establishment of a fully automated microtiter plate-based system for suspension cell culture and its application for enhanced process optimization, Biotechnol. Bioeng. 114 (2017) 113–121.
[25] K. Chaturvedi, S.Y. Sun, T. O'Brien, Y.J. Liu, J.W. Brooks, Comparison of the behavior of CHO cells during cultivation in 24-square deep well microplates and conventional shake flask systems, *Biotechnol. Rep. (Amst.)* 1–2 (2014) 22–26.
[26] S. Doig, F. Baganz, G.J. Lye, High-throughput screening and process optimisation, in: C. Ratledge, B. Kristiansen (Eds.), Basic Biotechnology, Cambridge University Press, 2006, pp. 289–306.
[27] W.A. Duetz, Microtiter plates as mini-bioreactors: miniaturization of fermentation methods, Trends Microbiol 15 (2007) 469–475.
[28] S.S. Ozturk, Engineering challenges in high density cell culture systems, Cytotechnology 22 (1996) 3–16.
[29] C.J. Huang, H. Lin, J.X. Yang, A robust method for increasing Fc glycan high mannose level of recombinant antibodies, Biotechnol. Bioeng. 112 (2015) 1200–1209.
[30] M. Micheletti, T. Barrett, S.D. Doig, F. Baganz, M.S. Levy, J.M. Woodley, G.J. Lye, Fluid mixing in shaken bioreactors: Implications for scale-up predictions from microlitre-scale microbial and mammalian cell cultures, Chem. Eng. Sci. 61 (2006) 2939–2949.
[31] M. Bausch, C. Schultheiss, J.B. Sieck, Recommendations for comparison of productivity between fed-batch and perfusion processes, Biotechnol. J. vol. 14 (2) (2019), https://doi.org/10.1002/biot.201700721.
[32] J.B. Sieck, C. Schild, J. von Hagen, Perfusion formants and their specific medium requirements, in: G. Subramanlan (Ed.), Continuous Biomanufacturing: Innovative Technologies and Methods, Wiley-VCH, 2018.
[33] M. Zhu, A. Goyal, D.L. Rank, S.K. Gupta, T. Vanden Boom, S.S. Lee, Effects of elevated pCO_2 and osmolality on growth of CHO cells and production of antibody-fusion protein B1: a case study, Biotechnol. Prog. 21 (2005) 70–77.
[34] W.T. Hsu, R.P. Aulakh, D.L. Traul, I.H. Yuk, Advanced microscale bioreactor system: a representative scale-down model for bench-top bioreactors, Cytotechnology 64 (2012) 667–678.
[35] D. Voisard, F. Meuwly, P.A. Ruffieux, G. Baer, A. Kadouri, Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells, Biotechnol. Bioeng. 82 (2003) 751–765.
[36] N. Hutchinson, N. Bingham, N. Murrell, S. Farid, M. Hoare, Shear stress analysis of mammalian cell suspensions for prediction of industrial centrifugation and its verification, Biotechnol. Bioeng. 95 (2006) 483–491.
[37] M. Brunner, P. Doppler, T. Klein, C. Herwig, J. Fricke, Elevated pCO2 affects the lactate metabolic shift in CHO cell culture processes, Eng. Life Sci. vol. 18 (3) (2018) 204–214, https://doi.org/10.1002/elsc.201700131.
[38] M.K.F. Wolf, V. Lorenz, D.J. Karst, J. Souquet, H. Broly, M. Morbidelli, Development of a shake tube-based scale-down model for perfusion cultures, Biotechnol. Bioeng. vol. 115 (11) (2018) 2703–2713, https://doi.org/10.1002/bit.26804.