CHAPTER FOURTEEN

Method to transfer Chinese hamster ovary (CHO) batch shake flask experiments to large-scale, computer-controlled fed-batch bioreactors

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Abstract

Chinese hamster ovary (CHO) cell cultures in industry are most commonly conducted as fed-batch cultures in computer-controlled bioreactors, though most preliminary studies are conducted in fed-batch shake flasks. To improve comparability between bioreactor studies and shake flask studies, shake flask studies should be conducted as fed-batch. However, the smaller volumes and reduced control in shake flasks can impact pH and aeration, which leads to performance differences. Planning and awareness of these vessel and control differences can assist with experimental design as well as troubleshooting. This method will highlight several of the configuration and control issues that should be considered during the transitions from batch to fed-batch and shake flasks to bioreactors, as well as approaches to mitigate the differences. Furthermore, if significant differences occur between bioreactor and shake flask studies, approaches will be presented to isolate the main contributors for these differences.

1. Introduction

Recombinant protein-based therapeutics provide treatment options for many diseases including cancer, diabetes, and autoimmune disorders. Chinese hamster ovary (CHO) cells are among the most commonly used mammalian cells for producing therapeutic proteins, also called biopharmaceuticals. CHO cells are used to manufacture more than 50% of biopharmaceuticals by sales with global biopharmaceutical sales over $180 billion per year (Walsh, 2018). CHO cells remain a successful workhorse in the biopharmaceutical industry largely due to post-translational modification capabilities that are analogous to human proteins (Dahodwala & Lee, 2019; Wuest, Harcum, & Lee, 2012; Xu et al., 2011). Additionally, the adaptability of CHO cells to serum-free conditions in suspension cultures allows for high cell densities and product concentrations that are unparalleled (Bandaranayake & Almo, 2014; Dahodwala & Lee, 2019; O'Brien, Mulukutla, Mashek, & Hu, 2020). Bioprocess optimization *via* maximizing cell density, productivity, and product quality continues to be an intense research focus in both academia and industry (Handlogten, Wang, & Ahuja, 2020; Hilliard & Lee, 2021; Mulukutla et al., 2019; Wasalathanthri et al., 2020).

Bioprocesses can be optimized by modifying feed and media formulations, manipulating process parameters, and exploring novel feed strategies (Craven, Shirsat, Whelan, & Glennon, 2013; Handlogten et al., 2017; Sumit et al., 2019). When manipulations are made to a process, it is crucial that the observed improvements are scalable, meaning that the characteristics

observed in small scale studies are observed in larger scale processes. For this reason, understanding how to perform quick, inexpensive, and applicable preliminary experiments can aid in identifying worthwhile manipulations to investigate in larger bioreactor systems (Sewell et al., 2019; Xu et al., 2016). Scale-up from shake flask experiments to larger bioreactors is a critical component of the bioprocess development pipeline (Goldrick et al., 2019; Janakiraman, Kwiatkowski, Kshirsagar, Ryll, & Huang, 2015; Sumit et al., 2019; Xing, Kenty, Li, & Lee, 2009; Xu & Chen, 2016) and is important for understanding the industrial implications of a newly developed process.

In this method, the process of taking CHO cells from batch shake flasks to computer controlled fed-batch bioreactor experiments will be described. The method first discusses important experimental design aspects in transitioning from batch shake flasks to fed-batch shake flasks. Then, the scale-up transition between fed-batch shake flasks and bioreactors will be discussed, as will approaches to account for discrepancies between the two scales and configurations.

2. Materials and equipment

2.1 Equipment

1. Air, clean, dry compressed (building supply or tank, regulated to <20 psi), check bioreactor manufacturer instructions for pressure requirements
2. ambr250 High Throughput or similar
3. Autoclave
4. Bioreactor tubing (Sartorius Stedium (001-2G44) or similar)
5. Bioreactor vessels (Sartorius Stedium (001-5G25) or similar)
6. Blood-gas analyzer (Radiometer or similar)
7. Bottle top filters (0.1 μm, sterile)
8. Cell counter, such as ViCell (Beckman) or similar
9. Centrifuge tubes (15 and 50 mL)
10. Disposable pipette tips (300 μL, Sartorius Stedium (001-2G72) or similar)
11. Drip catcher pads (Sartorius Stedium (001-2G94) or similar)
12. Feed bottles (sterile, 1-L and 175 mL, Sartorius Stedium (001-2G51, 001-2G50) or similar)
13. Filtered pipette tips (1–10 mL, sterile, Thermo Scientific (9402151) or similar)
14. Gas tanks (oxygen and carbon dioxide)

15. Glass autoclavable bottles (sterile, 1-L)
16. Metabolite analyzer (capable of measuring glucose, lactate, glutamine, glutamate, and ammonia, Cedex or similar)
17. Microcentrifuge tubes (1.5 mL) and rack
18. Osmometer (Advance or similar)
19. pH meter and calibration solutions
20. Pipettes (variable, 1–10, 10–100 and 100–1000 μL) with sterile tips
21. Sample collection plates
22. Serological pipettes (2, 10, 25 mL) and pipet aid
23. Shake flasks, unbaffled with vented lids (125-, 250- and 500-mL, Corning (431143, 431144, 431145) or similar)
24. Shaker/incubator or shaker compatible with CO_2 incubator (135–185 rpm, with clamps capable of holding 125-, 250- and 500-mL shake flasks)
25. Stir plate and stir bars
26. Syringes (2–10 mL) and syringe filters (0.2 μm)
27. Titer or protein product analysis (Cedex Bioanalyzer or similar)
28. Waste bags (Fisher Scientific or similar)

2.2 Chemicals

1. Antifoam solution (Cytvia (SH30897.01) or similar)
2. Cell culture-grade water (HyClone HyPure Water (Fischer Scientific (SH3052903) or similar))
3. Cell culture media (ActiPro Cytiva (SH31039.03) or similar)
4. Dextrose, D-Glucose, anhydrous powder (Fisher Scientific)
5. Feed media (HyClone CellBoost 7a and Fischer Scientific (NC1459945) or similar)
6. Hydrogen peroxide
7. Isopropyl alcohol (70%)
8. L-glutamine solution, 200 mM (Sigma-Aldrich (59202C-500ML) or similar)
9. Phosphate buffered saline
10. Sodium bicarbonate or sodium hydroxide

2.3 Cell lines

CHO cell line adapted to suspension culture expressing a desired recombinant protein. Examples are:

- CHO-K1 clone expressing the anti-HIV antibody VRC01 (IgG_1) donated by the National Institutes of Health (NIH).
- CHO DP-12 clone #1934 cells expressing anti-interleukin-8 (ATCC CRL-12445)

3. Step-by-step method details

CHO cells are widely used for biopharmaceutical production due to high production capabilities, scalability, and ability to grow in various serum-free medias (Lalonde & Durocher, 2017). Dr. Theodore Puck isolated the first population of CHO cells from a female hamster in 1957 (Dahodwala & Lee, 2019). Since then, all CHO cell lines have been derived from this original tissue (Wurm & Hacker, 2011). This method will outline transitioning from batch shake flasks to fed-batch shake flasks to fed-batch bioreactors. The recombinant CHO-K1 clone expressing the anti-HIV antibody VRC01 (IgG_1) will be used as an example, unless otherwise specified. "CHO VRC01" was donated by the National Institutes of Health (NIH).

3.1 Culture preparation

In order to have robust fed-batch cultures at shake flask scale, the cells must be maintained in exponential growth phase during scale-up. To ensure this behavior is reached, it is best to monitor the culture post-thaw for several passages. For example, the CHO cell shake flask batch shown in Fig. 1 took 2–3 passages to reach a stable exponential growth stage. The lag observed for passage 0 could, in some instances, be due to lower than normal cell viability when frozen, cells not in the exponential phase when frozen, residual dimethyl sulfoxide (DMSO) in the culture from freezing media, suboptimal freezing conditions, long storage, or storage in the liquid phase.

Tip: It is recommended to remove the freezing media 2 days after thaw, as it contains DMSO.

3.2 Feed preparation

To achieve higher cell densities and recombinant protein titers than can be achieved in batch shake flasks, fed-batch processes are used. In a fed-batch process, concentrated feeds provide amino acids, vitamins, salts, trace elements, and glucose (GE, 2016; Gorfien et al., 2013; Konakovsky, Clemens, Müller, Bechmann, & Herwig, 2017; Pan, Streefland, Dalm, Wijffels, & Martens,

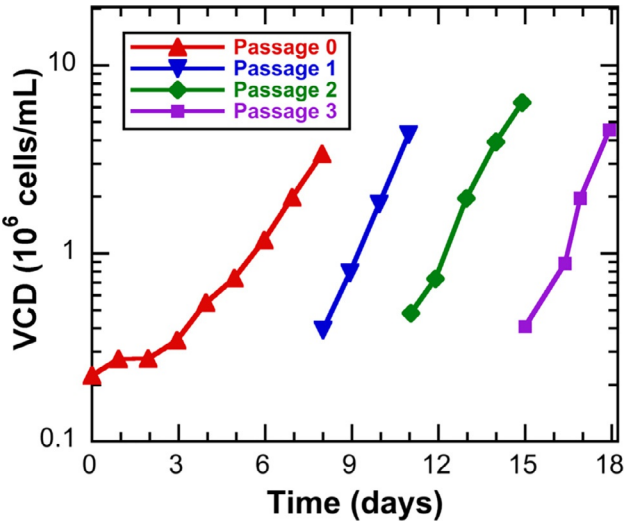

Fig. 1 Growth profile for a cell line adapting after thaw. CHO DP-12 clone #1934 cells (ATCC CRL-12445) adapted to BalanCD CHO (Irving Scientific) were thawed and monitored for several passages. Viable cell densities (VCD) were measured daily. Cells were cultured in a 5% CO_2, 37 °C incubator at a rotational speed of 180 rpm and 0.75 in throw.

2017). Most commercial feeds available are chemically-defined, animal-derived component-free, and have been optimized for high-yield recombinant protein production. Often, feeds consist of two components. For example, Cell Boost 7a contains glucose, surfactants, many amino acids, and vitamins, while Cell Boost 7b contains several basic pH amino acids. Cell Boost 7a is typically fed at 3–5% unit feed volume per unit culture volume (v/v) daily after Day 3. Cell Boost 7b is recommended to be fed at 10% of the volume of Cell Boost 7a. Prior to inoculation, the feeding strategy and the number and volume of samples should be decided as a variety of feeding schemes can be implemented for fed-batch fermentations. One example is daily feeding at constant percentage relative to the culture volume, such as 3%/0.3% v/v of Cell Boost 7a/7b. Another strategy increases the feeding volume as the viable cell density (VCD) increases and/or growth rate increases. For example, a pyramid feeding strategy that starts on Day 3 with Cell Boost 7a/7b could be Days 3–5, 3%/0.3%; Days 6–7, 4%/0.4%; Days 8–9, 5%/0.5%; Days 10–12, 4%/0.4%, Days 12–14, 3%/0.3%. This feeding scheme is beneficial as the nutrient levels increase with VCD and growth rate and then decreases while the VCD is still high, but the growth rate has slowed.

Table 1 Sampling and feeding plan for a fed-batch shake flask.

Day	Initial volume (mL)	7a feed added volume (mL)	7b feed added volume (mL)	Sampling volume removed (mL)	Final volume (mL)
0	70.0	0.0	0.00	2.0	68.0
1	68.0	0.0	0.00	2.0	66.0
2	66.0	0.0	0.00	2.0	64.0
3	64.0	1.9	0.19	2.0	64.1
4	64.1	1.9	0.19	2.0	64.2
5	64.2	1.9	0.19	2.0	64.3
6	64.3	1.9	0.19	2.0	64.4
7	64.4	1.9	0.19	2.0	64.5
8	64.5	1.9	0.19	2.0	64.6
9	64.6	1.9	0.19	2.0	64.7
10	64.7	1.9	0.19	2.0	64.8
11	64.8	2.0	0.20	2.0	65.0
12	65.0	2.0	0.20	2.0	65.2
13	65.2	2.0	0.20	2.0	65.4
14	65.4	2.0	0.20	2.0	65.6
Total feed volumes		**23.2**	**2.32**		

For a 250-mL shake flask the typical working volume is 70 mL. To ensure the working volume stays within the optimal range (50–70 mL), a daily sampling and feeding plan should be used. This plan also aids in determining the amount of each feed to be prepared prior to the shake flask study.

As shake flasks have limited optimal volume ranges for sufficient aeration, pre-planning is important. Table 1 outlines an example feeding and sampling plan for a fed-batch 250-mL shake flask with and initial media volume of 70 mL. In this example case, the optimal working volume of a 250 mL shake flask is 50–70 mL. Cell Boost 7a/7b are planned for constant daily feeding at 3%/0.3% (v/v) starting on Day 3. It was planned that 2 mL samples would be taken daily for cell counts and metabolite analysis, such as glucose, lactate, and titer measurement. A similar approach can be used for other feeding schemes. Although Cell Boost 7a contains glucose, additional glucose is often required to avoid glucose depletion. For both fed-batch shake flasks

and computer-controlled bioreactors, it is typical to supplement glucose such that glucose concentrations return to 6–9 g/L each day (Fan et al., 2015). Since glucose stock solutions typically contain 200–450 g/L, the volume added due to glucose supplementation is less than 2% of the total volume. Using this preplanning approach, the volume in the fed-batch shake flask can be estimated throughout the culture, and the volume maintained within the optimal shake flask volume range.

3.3 Shake flask volume considerations

Shake flask volume is an important consideration for sampling and feeding, and it can also effect aeration. Proper aeration can lead to higher cell densities, better productivity, and reduced waste products (Betts et al., 2014; Nienow, 2015). In order to determine the impact shake flask volume has on oxygen availability, the partial pressure of dissolved oxygen (pO_2, mmHg) was measured during batch cultures in 125-, 250-, and 500-mL shake flasks containing initial volumes of 30, 70, and 150 mL, respectively. A blood gas analyzer was used to obtain offline dissolved oxygen measurements. Fig. 2 shows the cell growth and dissolved oxygen profiles. The growth rates and cell viabilities for Days 0–3 were not statistically different, indicating that the shake flask volume would not have an impact on overall culture performance when limited to 3-day passages typical of scale-up. After Day 3, cell growth was slightly better for the 500-mL flasks, which were also observed as having slightly lower pO_2. Even though pO_2 of the 500-mL shake flasks were statistically lower ($p \leq 0.05$), pO_2 was still above the critical oxygen level required for CHO cells (~35 mmHg or 20% saturation) (Heidemann, Lütkemeyer, Büntemeyer, & Lehmann, 1998). Hence, shake flasks between 125- and 500-mL in volume performed similarly for 3-day cultures, but for longer cultures, larger shake flasks potentially could provide better growth conditions, assuming pO_2 stays above the critical level.

3.4 Experimental additives considerations

In addition to testing parameters related to media and feed manipulations, it may be desirable to supplement cultures with other compounds to better understand how these components impact the culture. Example additions could include increased salt concentrations, lactate, and ammonia (Chen & Harcum, 2005; Chitwood et al., 2021; Freund & Croughan, 2018; Ha, Kim, & Lee, 2014; Jinyan et al., 2019; Pereira, Kildegaard, & Andersen, 2018; Schmelzer, deZengotita, & Miller, 2000). When preparing concentrated

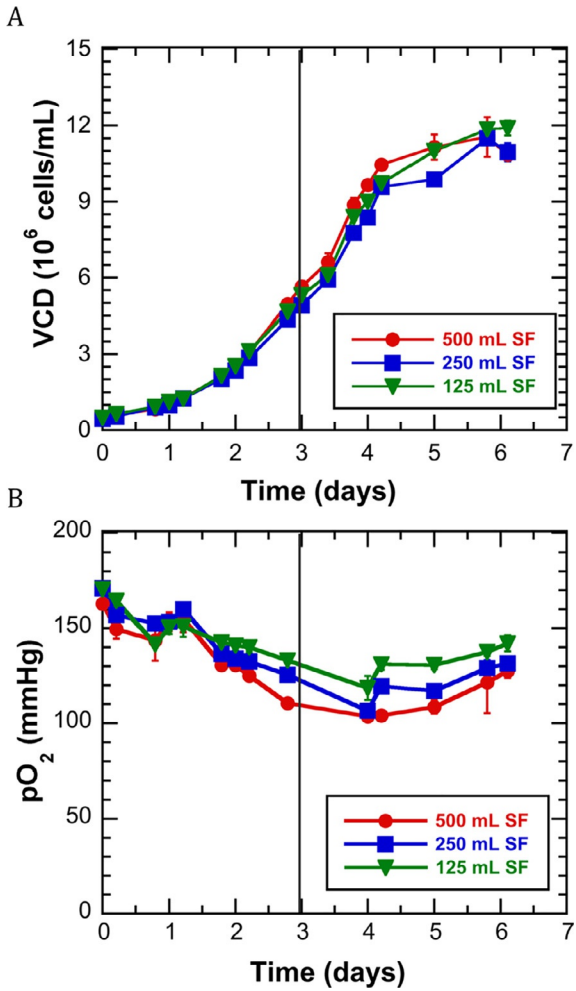

Fig. 2 Effect of culture volume on growth and oxygenation for batch shake flask cultures. Baffled shake flasks of 125-, 250- and 500-mL were used with working volumes of 30, 50 and 70 mL, respectively. The shaker throw diameter was 0.75 in, and the rotational speed was 135 rpm. (A) VCD and (B) dissolved oxygen (pO_2). The vertical line at 3 days indicates the time when cells would be typically passaged. Dissolved oxygen saturation is approximately 160 mmHg. Error bars represent standard deviations ($N = 2$).

solutions, the concentration should be as high as possible to minimize volume changes from the addition. If potentially harmful compounds are being added, it is advisable that pilot experiments be performed in shake flasks prior to bioreactors. These pilot experiments can determine the additive amount for the desired outcome, confirm that significant cell death does not occur, and test

dosing approaches. Further, concentrated additives can impact osmolarity and/or pH. Therefore, osmolarity and pH should be assessed throughout the cultures. If substantial pH and osmolarity deviations are observed, additional control cultures should be considered to assess these impacts separately. Also, it is recommended to wait a minimum of 12 h post-inoculation to add concentrated additives. This will ensure the cultures are established and synchronized with respect to growth prior to the potentially stressful addition. Further, dosing can include a single addition or subdivided additions added discretely over equal time intervals. For example, the effects of lactate additions on CHO VRC01 were explored in batch shake flasks prior to fed-batch shake flasks, and computer-controlled bioreactors. Lactate was added either as a single 25 mM addition, or as divided 10 mM additions (30 mM total). The growth, glucose, and lactate profiles are shown in Fig. 3. Batch and fed-batch shake flasks can provide an inexpensive alternative to assess the effects of a particular addition on cell growth and productivity prior to beginning more costly experiments.

3.5 Rotational speed and throw diameter considerations

1 Throw diameter and rotational speed are known to impact aeration for CHO cell cultures, especially fed-batch shake flasks with higher VCDs (Mora et al., 2018; Qian et al., 2014). The widely accepted rule of thumb is based on maintaining the centrifugal force between shakers of different throw diameters (Klöckner, Diederichs, & Büchs, 2013; Takahashi & Aoyagi, 2018). The centrifugal force rule of thumb has been demonstrated to work well with bacterial systems (Henzler & Schedel, 1991) and is commonly used for mammalian cells; however, limited literature supports the use of the centrifugal force rule of thumb as the best approach for mammalian cells oxygenation in shake flasks (Hacker, Durrer, & Quinche, 2018; Shiue, Chen, Jeng, Zhu, & Leggett, 2020). For the centrifugal force approach, the forces (F) are maintained as equal between the throw diameters. For example, $F_{throw1} = F_{throw2}$, where F is the centrifugal force (kgm/s^2) for the respective throw diameters. Further, force can be defined as $F = ma$ where m is the mass of a cell (kg) and a is the centrifugal acceleration (m/s^2), on the shaker. Centrifugal acceleration can be defined as $a = (v^2/r)$, where v is the shaker velocity (m/s) and r is the throw radius (m). Fig. 4 shows the relationship of these variables graphically. The velocity of the cells on a shaker can be represented by $v = \omega r$, where ω is the angular velocity (radians/s) and r is the throw radius (m). The rotational speed (N, rpm) is defined as $\omega = 2\pi N/60$.

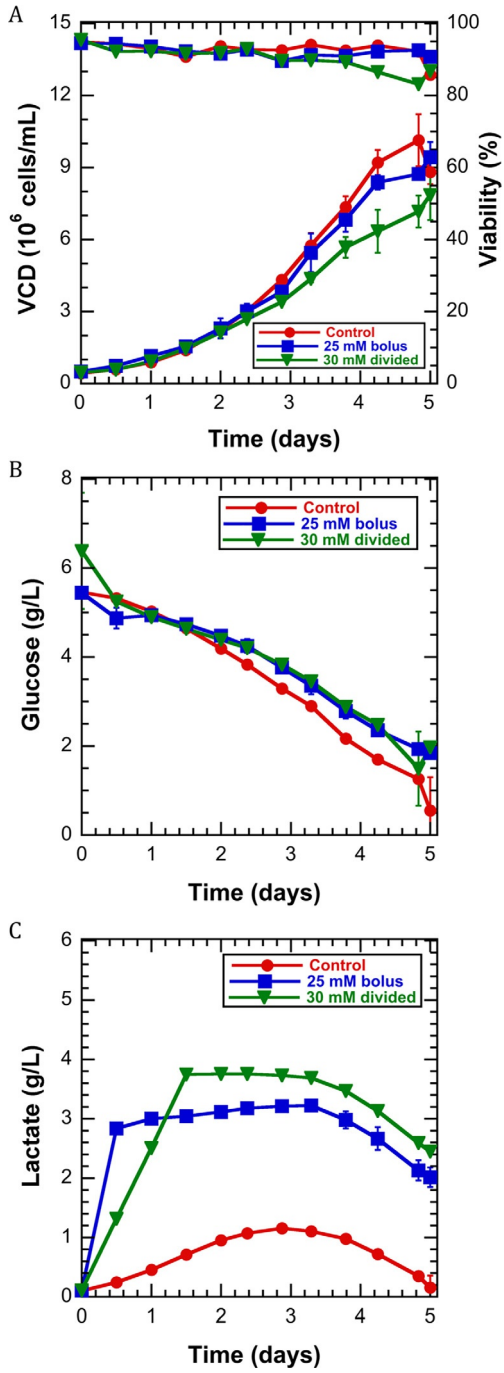

Fig. 3 Effect of lactate addition on growth of CHO VRC01 in batch shake flask cultures. (A) VCD and viability, (B) glucose, and (C) lactate. Error bars represent standard deviations ($N = 2$).

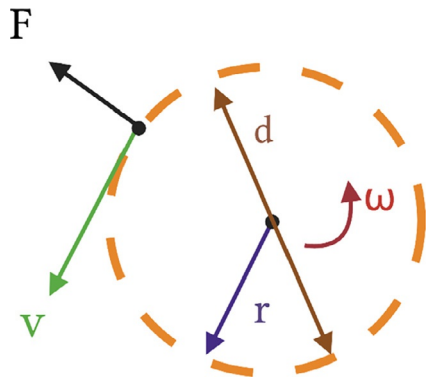

Fig. 4 Comparison of centrifugal force (**F**) and velocity (**v**) vectors. Throw radius (**r**, m), throw diameter (**d**, m), and rotational speed (ω, radians).

Thus, to determine the necessary rotational speed, based on constant the centrifugal force rule of thumb in terms of throw diameter, d_{throw}, can be written as Eq. (1).

$$N_{throw2} = N_{throw1}\sqrt{\frac{d_{throw1}}{d_{throw2}}} \tag{1}$$

However, observations of CHO cell cultures suggest this relationship does not provide adequate aeration when used for fed-batch CHO cell cultures in shake flasks, likely due to the higher achieved cell densities than obtained in batch shake flask cultures. Figs. 3 and 5 highlight the increased VCD that can be achieved for fed-batch relative to batch studies for the same cell line with the same batch media. In Fig. 5, the growth and titers are compared for different throw diameters with the same- and an increased rotational speed. The increased rotational speed was based on the centrifugal force rule of thumb. The growth rates were similar for the three throw diameter and rotational speed combinations. However, the titers at Day 12, for the 0.75 in throw/135 rpm cultures were lower than the 1 in throw/135 rpm cultures ($p \leq 0.05$). The 0.75 in throw/161 rpm culture titers were not different from the other two conditions ($p > 0.05$). The lactate was observed to increase late in the cultures for all throw and rotational settings, which suggests there might have been oxygenation limitations at all throw and rotational settings late in the cultures (data not shown). These observations led us to explore alternatives to the centrifugal force based calculations for calculating optimal rotational speed and throw diameter considerations.

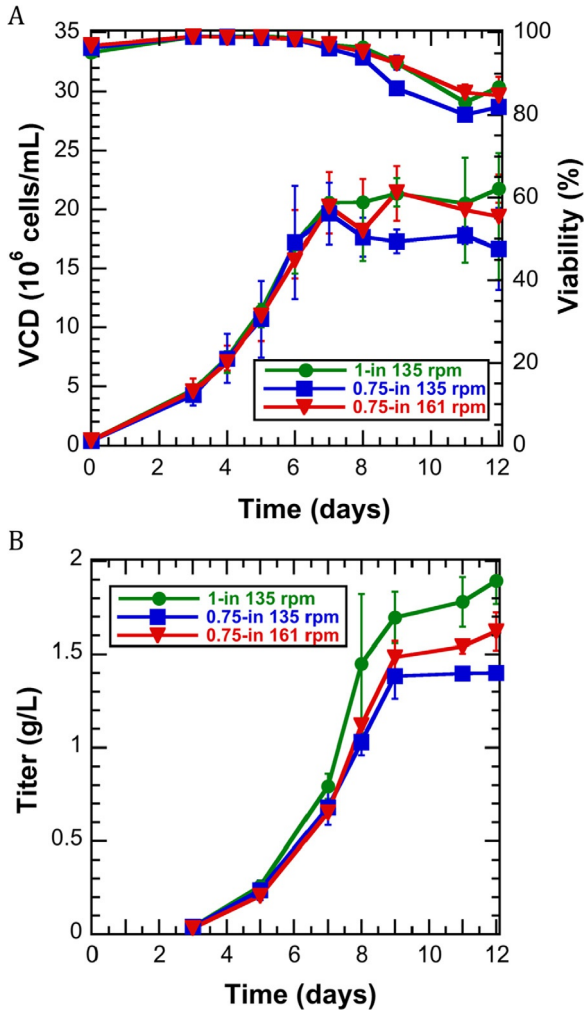

Fig. 5 Comparison of shaker speed and throw diameter on CHO VRC01 growth and productivity. Shaker throw diameters and rotational speeds are 1 in./135 rpm, 0.75 in./135 rpm, and 0.75 in./160 rpm. CHO VRC01 were cultured in ActiPro media with feeding. Cell Boost 7a/7b were fed daily at 3%/0.3% beginning on Day 3. (A) VCD and viability, and (B) titer. Titers were evaluated using an Octet titer analyzer (Fortebio). Error bars represent standard deviations ($N = 2$).

2 For CHO cells, it may be better to use a rule of thumb based on constant fluid velocity for aeration in fed-batch shake flasks. Essentially, a constant velocity between throw diameters would be given by $v_{throw1} = v_{throw2}$. A comparison of the centrifugal force (F) and velocity (v) are shown in

Fig. 4. If constant velocity is used, Eq. (2) describes the relationship between rotational speed (N_{throw}) and the throw diameter (d_{throw}) as

$$N_{throw2} = N_{throw1}\left(\frac{d_{throw1}}{d_{throw2}}\right) \tag{2}$$

To examine the constant velocity rule of thumb, CHO cells were cultured using a 0.75 in throw diameter shaker at 135 and 180 rpm. As shown in Fig. 6, the cell growth profiles were significantly different when using the different shaker speeds, where the higher speed resulted in better growth. Further, the pO_2 profiles demonstrated that both cultures consumed oxygen rapidly; however, the cultures at the higher rotational speed did not appear to be as oxygen-deprived. More importantly, the titer was higher for the cultures at the higher rotational speed ($p \leq 0.05$). Further, the relative increase in titer was the same for the cultures shown in Figs. 5 and 6. For the cultures shown in Fig. 5, the fold increase in titer between the 0.75 in and 1 in throw diameters at the same rotational speed was 1.36 ± 0.093-fold. Likewise, the fold increase for the culture shown in Fig. 6, and due to the increase rotational speed was 1.32 ± 0.045-fold (Hayya, Armstrong, & Gressis, 1975). Using a unpaired *t*-test, these fold-changes were not different ($p > 0.05$). These results indicate that for shakers with 0.75 in throws, cultures should have rotational speeds of 180 rpm to better match data from 1 in throw diameter shakers at 135 rpm.

Note: The absolute titer values differences shown for the centrifugal force and velocity demonstrations is due to different analysis tools, Octet and Cedex, respectively.

Note: The growth profile differences shown in Figs. 5 and 6 are the result of two different nutrient feeding strategies. Namely, the cultures in Fig. 5 used a constant addition feeding strategy, while the cultures shown in Fig. 6 used pyramid feeding strategy, which provides higher nutrient additions.

3.6 Experimental design considerations during scale-up

During the scale-up pipeline for bioreactors, it is important to generate sufficient cells. A typical shake flask will have 70–150 mL working volumes, while most bioreactors are much larger. For example, if the bioreactor system is an ambr250 high throughput system, the working volume of the ambr250 vessel is 200–250 mL, thus 12 vessels would result in a 2.4–3.0 L

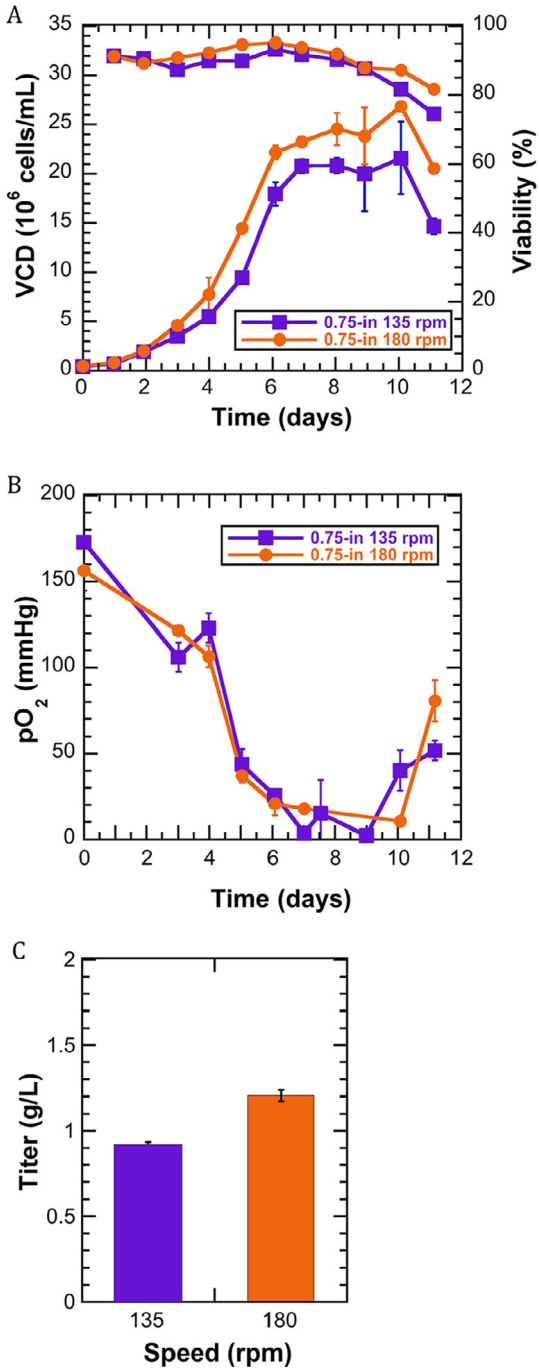

Fig. 6 Comparison of growth, oxygenation, and productivity for CHO VRC01 in fed-batch shake flasks with different rotational speeds. Shaker rotational speeds of 135 rpm and 180 rpm were compared at 0.75 in. throw diameters. A pyramid fed-batch feeding protocol was used, where Cell Boost 7a/7b were initially fed on Day 3. (A) VCD and viability, (B) pO_2, and (C) titer. Titers were evaluated using a Cedex Bioanalyzer. Error bars represent standard deviations ($N = 2$).

total working volume. Thus, approximately 300 mL batch shake flask culture volume would be required to inoculate a 12-vessel ambr250 study from a common inoculum. In order to achieve sufficient cells in the bioreactors ($4–5 \times 10^5$ cells/mL) without a lag phase, exponential phase cultures are required. Figs. 1 and 2 highlight that consistent passages with exponential phase growth are greatly decrease variability in the bioreactors. Correspondingly, larger bioreactors or more ambr250 vessels would require higher batch shake flask volumes. WAVE rocking bag bioreactors (up to 25 L) are commonly used for large bioreactor inoculums, again with the goal of maintaining the cells in the exponential phase during scale-up (Ghasemi, Bozorg, Rahmati, Mirhassani, & Hosseininasab, 2018; Xu et al., 2020). The time to expand from the frozen working cell bank to bioreactor inoculation can take weeks. For this reason, it is beneficial to design experiments that match bioreactor conditions to shake flask conditions to best understand how inherent differences in the vessel contribute to observed differences in cell culture.

In order to better understand the operational difference between shake flasks and computer-controlled bioreactors, Fig. 7 contains a comprehensive list of bioreactor variables. The impact of these variables on culture outcome will be discussed, as well as approaches to isolate root causes for differences in culture performance between fed-batch shake flasks and fed-batch bioreactors.

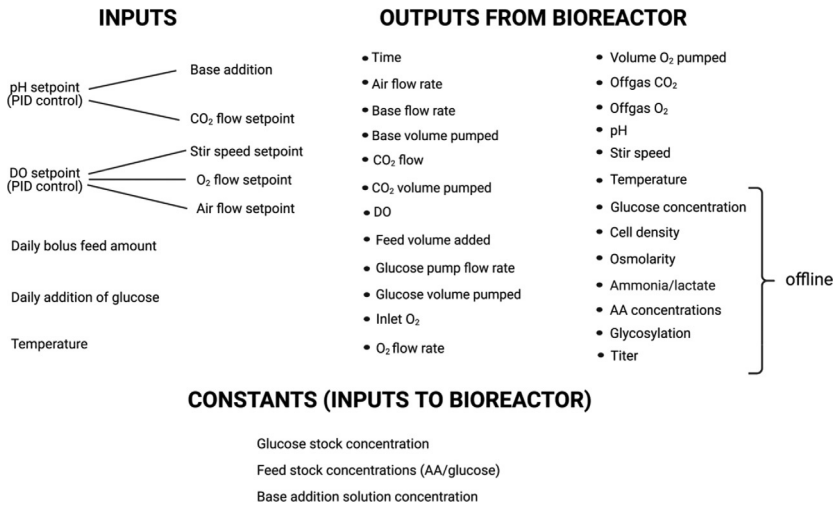

Fig. 7 Overview of bioreactor inputs and outputs. Inputs are variables which can be controlled by experimental design or setpoints. Outputs are variables that are typically measured online or offline.

1 In a shake flask, pH control is limited to the carbon dioxide equilibrium between the bicarbonate buffered media and the ambient 5% CO_2 gas in the incubator (Goudar et al., 2007). In contrast, in a bioreactor, pH control can be set to a specific value or to a narrow range. The pH is then manipulated in the bioreactor by CO_2 sparging rates and base-additions (sodium bicarbonate, sodium hydroxide, or carbonate solutions).

Normally, the outcomes in the bioreactor are better than that of the fed-batch shake flask due to improved control of culture conditions, such as pH and pO_2 (Nienow, 2014). So, when it was observed that the cell specific productivity (q_p) results for the fed-batch shake flasks were not achieved in the fed-batch bioreactors (Fig. 8), further investigation into the root cause was undertaken.

In this example, a standard feeding scheme was compared with an experimental feeding scheme (Fig. 8). The fed-batch shake flask cultures with the experimental feeding scheme demonstrated higher overall titer and cell specific productivity (q_p). However, when these feeding strategies were implemented in the ambr250 bioreactors, the reverse outcomes were observed. The overall titer and cell specific productivities were significantly lower for the experimental feeding strategies. Based on the schematic in Fig. 9, the possible causes were outlined. Mainly, the pH, vessel configuration, and sparging were identified as likely causes of the differences. Operationally, the easiest to examine was the pH control differences. As controlling pH in a shake flask is difficult, it was decided to mimic the pH profile of a shake flask in the ambr250. The pH profile for fed-batch shake flasks was characterized using a blood gas analyzer. The pH was observed to vary from pH 6.8–7.3. Typically, the bioreactor pH is held to pH 7.0 ± 0.1. The shake flask pH profile was then used with the ambr250. The outcomes from the bioreactor using the experimental feeding strategy are shown in Fig. 8. The cell specific productivity of the pH-matched experimental feeding strategy bioreactor significantly increase over the bioreactors with the standard and experimental feeding strategies.

2 If the pH-matching approach outcomes had not increased and become closer to the shake flask outcomes, the next difference to examine would be the sparging differences. A bioreactor has the capability to bubble gases (typically air, oxygen, and carbon dioxide for cell culture) to control the pO_2 and pH. The gas flow rates can be manipulated. If the results suggest cell lysis due to bubble breakage, overlay gassing can be used in the bioreactor to eliminate directly adding gas bubbles. The overlay gassing approach will significantly decrease the volumetric mass transfer coefficient (k_La) (Nienow, 2015), which might decrease pO_2 in the bioreactor.

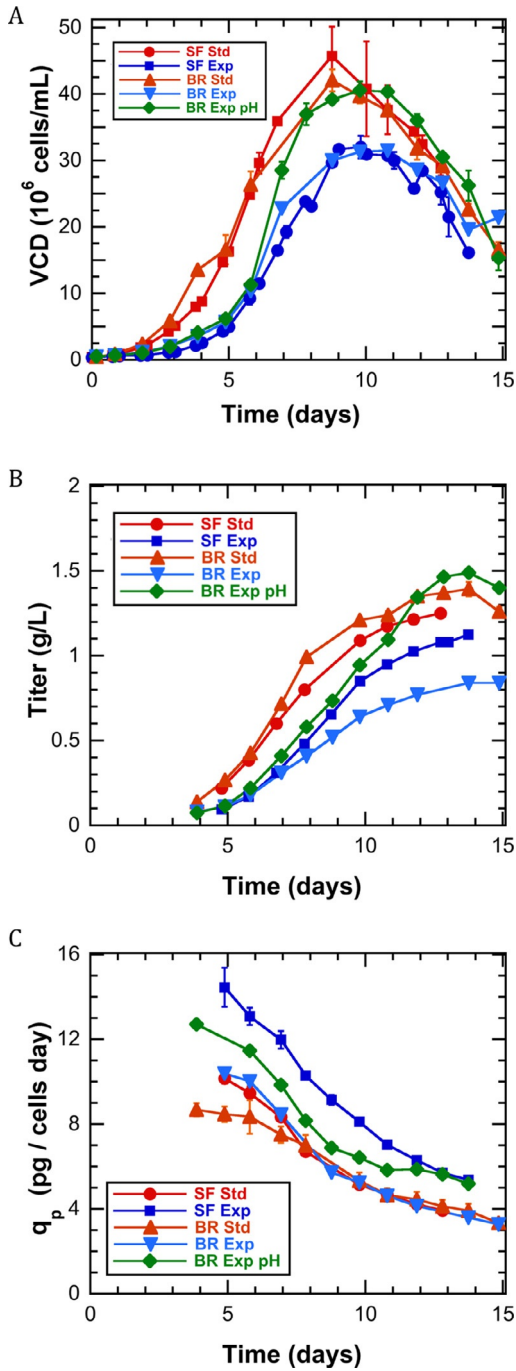

Fig. 8 Effect of experimental feed on CHO VRC01 growth and productivity. Shake flasks received either a standard 3%/0.3% (v/v) daily feeding protocol (SF Std) or an experimental feeding protocol (SF Exp). Bioreactors received either a standard pyramid feed (BR Std) or the same experimental feeding protocol (BR Exp). Bioreactors that had pH matched to shake flask culture are indicated as "BR Exp pH." (A) VCD, (B) titer, and (C) cell specific productivity (q_p). Error bars represent standard deviations ($N = 2$).

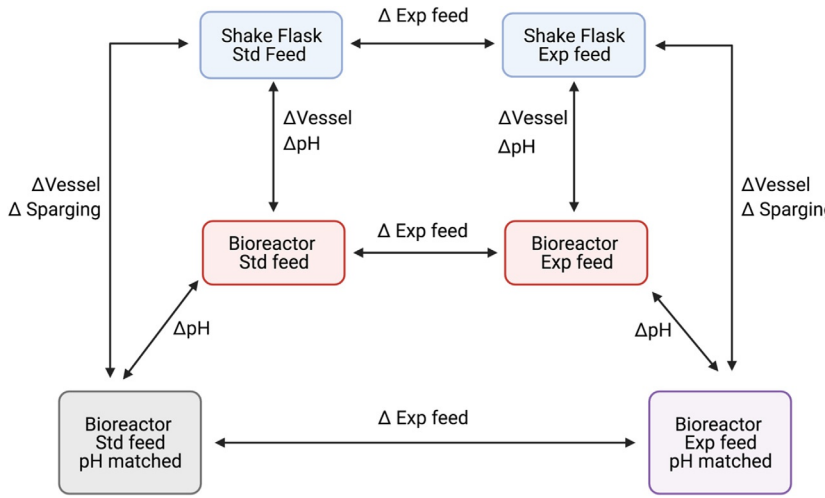

Fig. 9 Flow diagram for troubleshooting unexpected differences between fed-batch shake flasks and computer-controlled bioreactors. A standard (Std) fed-batch feeding strategy was compared to an experimental (Exp) feeding strategy. In this example, the pH was evaluated as a potential contributing factor impacting study results.

As fed-batch shake flasks do not have gas sparging, mimicking the pO_2 profile of the shake flasks would aid in determining the effects of sparging independently of the dissolved oxygen. A blood gas analyzer can be used to obtain daily pO_2 measurements in the fed-batch shake flasks, similar to the data for batch shake flasks presented in Fig. 2. Thus, a similar approach to the pH-matching can be investigated for sparging concerns.

3 Finally, the vessel physical configuration should be investigated if pH and sparging did not reveal the root cause of unexpected outcomes. An inherent difference between shake flasks and bioreactors is the agitation mechanism. Bioreactors are typically equipped with impellers which can create high shear at the impeller tips. Previous studies of shear effects with CHO cells have indicated this is not a major cause of cell damage (Hu, Berdugo, & Chalmers, 2011; Nienow, 2006). However, if other causes cannot explain the observed outcomes, approaches to address shear can include using only sparging to mix the culture in the bioreactor or using surface aeration (also call overlay gassing) with very low impeller speeds. It is more advisable to use surface aeration first, as it has been suggested that bursting air bubbles can cause damage to CHO cells (Walls et al., 2017).

As these three troubleshooting approaches have demonstrated, it is easier to make the bioreactor mimic a shake flask, than the reverse. Further,

developing testable approaches, such as the schematic shown in Fig. 9 outlines, is fundamental to determining the root causes of differences and deciphering unexpected outcomes.

4. Advantages

Understanding fed-batch shake flask culture conditions aids in the transition between small scale vessels and larger scale computer-controlled bioreactors. A well characterized scale up for bioreactor seeding will potentially reduce issues with reproducibility and even aid in consistent product quality. Going through the experimental design process described will allow for more robust understand of major contributors to cell culture performance.

5. Limitations

Not all differences between fed-batch shake flasks and bioreactors will have a clear method for testing during troubleshooting.

6. Safety considerations and standards

As recombinant CHO cells are being cultured, one must follow NIH guidelines for working with recombinant organisms.

Note: Bacterial culture cell densities are most often measured by optical density, where the optical density can be correlated to dry cell mass, *i.e.*, on the spectrophotometer Spectronic 20 (Genesys), a reading of 5 OD_{600} corresponds to 2.5 g dry cell weight per L (g dcw/L) (Baig et al., 2014). Typically, *Escherichia coli* reach maximum cell densities in a shake flask corresponding to 3–5 OD_{600} with minimal media (Korz, Rinas, Hellmuth, Sanders, & Deckwer, 1995) or LB media with 0.2% glucose media (Sharma, Campbell, Frisch, Blattner, & Harcum, 2007), as this is when oxygen is normally depleted using 250 rpm (0.75 in throw). As *E. coli* are considered to have a mass of approximately 1 pg per cell (Milo & Phillips, 2015), a shake flask of *E. coli* at 5 OD_{600} corresponds to 2.5×10^{12} cells/L (2.5×10^{9} cells/mL and 2500 million cells/mL). In a shake flask, CHO cell cultures can reach about 20–25 million cells/mL at 180 rpm (0.75 in throw). CHO cells are about 329 pg each (Templeton, Dean, Reddy, & Young, 2013), such that 25 million cells/mL would correspond to approximately 8.2 g dcw/L.

The different rotational speeds, 130–180 rpm for CHO cells and 200–250 rpm for *E. coli*, would not increase the *E. coli* biomass, as the

volumetric mass-transfer coefficient (k_LA) is proportional to the rotational speed (Klöckner & Büchs, 2012). Since the oxygen uptake rate (OTR) is proportional to kLa, and the oxygen uptake rate (OUR) equals OTR at steady-state, the maximum cell densities, 8.2 and 2.5 g dcw/L for CHO cell and *E. coli*, respectively, represent the relative OUR for each cell type. Thus, on total biomass basis, the oxygen demand is lower for CHO cells, since a higher biomass concentration can be supported, regardless of the exact rotation speed (130–250 rpm). Conversely, on a cell concentration basis, the oxygen demand per cell is higher for CHO cells.

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