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ARTICLE

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Developing an ultra-intensified fed-batch cell culture process with greatly improved performance and productivity

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Abstract

Intensified fed-batch (IFB), a popular cell culture intensification strategy, has been widely used for productivity improvement through high density inoculation followed by fed-batch cultivation. However, such an intensification strategy may counter-productively induce rapidly progressing cell apoptosis and difficult-to-sustain productivity. To improve culture performance, we developed a novel cell culture process intermittent-perfusion fed-batch (IPFB) which incorporates one single or multiple cycles of intermittent perfusion during an IFB process for better sustained cellular and metabolic behaviors and notably improved productivity. Unlike continuous perfusion or other semi-continuous processes such as hybrid perfusion fed-batch with only early-stage perfusion, IPFB applies limited times of intermittent perfusion in the mid-to-late stage of production and still inherits bolus feedings on nonperfusion days as in a fed-batch culture. Compared to IFB, an average titer increase of ~45% was obtained in eight recombinant CHO cell lines studied. Beyond IPFB, ultra-intensified IPFB (UI-IPFB) was designed with a markedly elevated seeding density of $20–80 \times 10^6$ cell/mL, achieved through the conventional alternating tangential flow filtration (ATF) perfusion expansion followed with a cell culture concentration step using the same ATF system. With UI-IPFB, up to ~6 folds of traditional fed-batch and ~3 folds of IFB productivity were achieved. Furthermore, the application grounded in these two novel processes showed broad-based feasibility in multiple cell lines and products of interest, and was proven to be effective in cost of goods reduction and readily scalable to a larger scale in existing facilities.

KEYWORDS

CHO cell culture, intensified fed-batch, intermittent-perfusion fed-batch, perfusion culture, process intensification, ultra-intensified intermittent-perfusion fed-batch

Abbreviations: ATA, aurintricarboxylic acid; ATF, alternating tangential flow filtration; CFB, concentrated fed-batch; CHO, Chinese hamster ovary; CM, continuous manufacturing; COG, cost of goods; CSPR, cell specific perfusion rate; HMW, high molecular weight species; IFB, intensified fed-batch; IPC, intensified perfusion culture; IPFB, intermittent-perfusion fed-batch; IVCD, the cumulative integral of viable cell density; mAb, monoclonal antibody; Qp, cell specific productivity; ROS, reactive oxygen species; SUB, single use bioreactor; TFB, traditional fed-batch; UI-IPFB, ultra-intensified intermittent-perfusion fed-batch; VCD, viable cell density; VVD, vessel volume per day; VVT, vessel volume each time.

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Shaoxun Xiang and Jinliang Zhang contributed equally to this work.

1 | INTRODUCTION

Current process development for mammalian cell culture highlights the need for yield improvement and cost of goods (COG) reduction to achieve broader patient access, and in the meantime better process economy in industrial biomanufacturing (Yang et al., 2019). For such a purpose, process intensification using a variety of cell culture approaches has been extensively studied to maximize productivity while maintaining comparable quality profiles (Xu et al., 2020a; Yang et al., 2016; Zhou et al., 2021). Based on how the cell cultivation is operated during production, these intensification strategies could be classified into two major categories: fed-batch with bolus feeding of nutrients, and perfusion via continuous medium exchange through a cell retention device such as the alternating tangential flow filtration (ATF) system. Owing to the operational simplicity and scalability, fed-batch has been one of the most widely adopted approaches in CHO-based bioproduction. Evolved from fed-batch, IFB was developed with the purpose to produce a higher magnitude of products through high density inoculation in production achieved by either perfusion or enriched batch culture in seed expansion (Xu et al., 2020a; Yang et al., 2014; Yongky et al., 2019).

On the other hand, perfusion cultures are regarded as a common option when a produced entity (e.g., enzymes) requires special attentions on quality and stability during production, or a large market demand is anticipated (Bielser et al., 2019; MacDonald et al., 2022). Under the classification of perfusion cultures, two process derivatives concentrated fed-batch (CFB) and intensified perfusion culture (IPC) were developed by applying continuous media exchange without cell bleeding to eliminate product loss during constant cell density control in conventional perfusion cultures. In a CFB culture, by applying an ATF filter with a small pore size (e.g., 50 kD), the produced products, such as monoclonal antibodies (mAb), could be retained within production vessels so that the downstream processing could be simply operated in a batch mode (Yang et al., 2016). Many studies have shown that by using the CFB process, upstream productivities could be improved significantly with the highest titer reported to be 25–30 g/L in a 14–21 days of production (Chon & Zarbis-Papastoitsis, 2011; DePalma, 2010; Schirmer et al., 2010). The selection of ATF filters differentiates CFB from IPC in which a larger pore size (e.g., 0.2 μM) is often chosen to allow continuous product harvesting. The continuous harvest could contribute to better product quality due to low product residence time, improved downstream resin efficiency, and meanwhile the continuous removal of process-related impurities, such as host cell proteins, from production vessels, contributing to superior cell performance and cumulative productivity. As high as 85.86 g/L of protein production was documented by using the IPC upstream process (Zhou et al., 2020; Zhou et al., 2021). Different from fed-batch cultures, the CFB or IPC process often involves particular considerations for perfusion-related process controls including an appropriate perfusion scheme that fits for an optimized perfusion medium, and the replacement of ATF filters to minimize fouling or protein retention (MacDonald et al., 2022; Maria et al., 2023; Wong et al., 2022; Zhang et al., 2020). Given the complexity of perfusion process, even though the concept of continuous manufacturing has emerged rapidly recently, the development complexity as well as facility readiness and fit can not be underestimated in both upstream and downstream process development (DePalma, 2009; DePalma, 2010; Schirmer et al., 2010).

Built on the fed-batch process, IFB cultures increased productivity through piling up initial cell density with either perfusion or enriched batch cultures in seed expansion (N-1). Typically, a seeding density of $2–10 \times 10^6$ cells/mL was reported with the highest density reaching 20×10^6 cells/mL. Both perfusion and enriched batch approaches were applied in N-1 previously to inoculate an IFB production culture, which gave rise to comparable productivity under a same seeding density of 5×10^6 cell/mL (Xu et al., 2020a; Yongky et al., 2019). Entering the production stage, the benefit of IFB can only be established on the premise that the status of cells, such as cell density and viability and cell specific productivity, could be sustained till a desirable advantage in volumetric productivity can be discerned. However, maintaining cell status in such a high density culture appeared to be challenging and cell line dependent (Stepper et al., 2020; Xu et al., 2020a; Yang et al., 2014; Yongky et al., 2019), as many studies have observed that some toxic cellular metabolites, such as lactate and ammonia, and metabolic by-products like reactive oxygen species (ROS), would exert a chain of deteriorating effects on cellular ecosystem and metabolic disorder (Graham et al., 2021; Handlogten et al., 2018; Komuczki et al., 2022; Pereira et al., 2018; Zhou et al., 2023). Consequently, extensive developmental efforts particularly on media or process optimization were often required to achieve a competitive productivity (Stepper et al., 2020; Xu et al., 2020a). Unfortunately, no universal solution has been published so far to fundamentally rescue the undesirable toxic by-product accumulation and poor production performance often occur during IFB development.

In an effort to improve IFB performance, a novel cell culture process intermittent-perfusion fed-batch (IPFB), a hybrid of IFB and perfusion cultures, was developed in this study (Figure 1a). More specifically, by performing intermittent perfusion in one or several blocks of time during the mid-to-late stage of an IFB production, and in the meantime maintaining the similar operation of IFB, such as the high seeding density ($5–15 \times 10^6$ cell/mL) inoculation and bolus feeding in nonperfusion days, the new process enabled sustained process performance in the same or extended culture duration. The IPFB process is different from a previously reported hybrid perfusion fed-batch in a sense that the hybrid perfusion fed-batch initiated perfusion in the early cell exponential stage solely to accumulate cell biomass from a low-initial seeding density (Hiller et al., 2017), whereas IPFB leveraged media exchange to remove toxic by-products, provide refreshed culture environment and extend productivity under the context of an IFB process. Meanwhile, compared to continuous CFB/IPC processes, IPFB utilizes limited cycles of intermittent perfusion to achieve desirable process performance with more cost-effective and operation-friendly media usage in commercial manufacturing. A detailed comparison of different cell culture modes was summarized in Table 1 and illustrated Figure 1c.

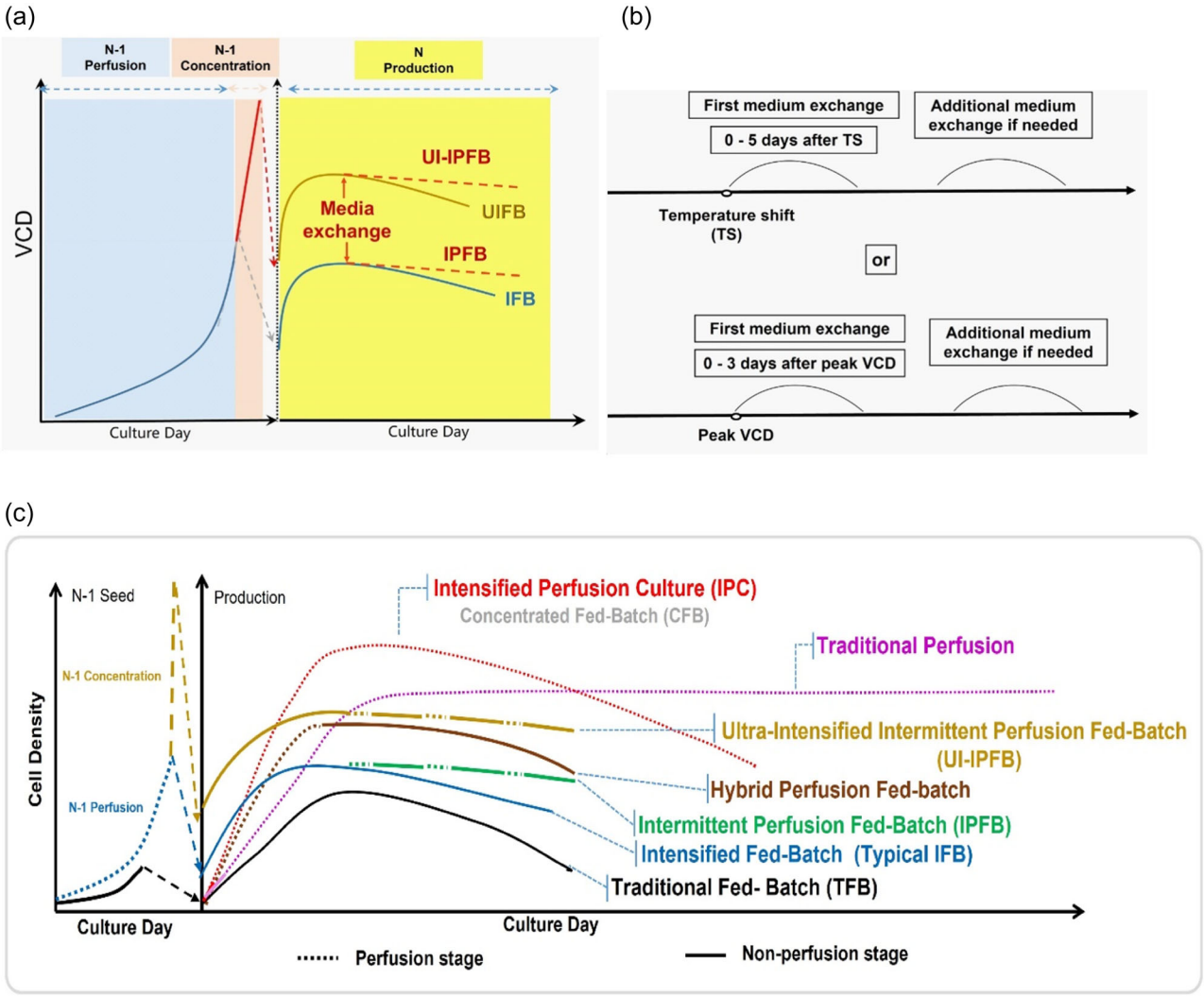

FIGURE 1 Schematics of intermittent-perfusion fed-batch (IPFB) and ultra-intensified intermittent-perfusion fed-batch (UI-IPFB) processes in comparison to intensified fed-batch (IFB) and ultra-intensified fed-batch (UIFB) (a), recommended media exchange schedules (b) and a comparison of different culture modes including traditional fed-batch (TFB), IFB, IPFB, UI-IPFB, traditional perfusion, intensified perfusion culture (IPC)/concentrated fed-batch (CFB) and hybrid perfusion fed-batch (c).

To further extend the potential of IPFB, N-1 cell culture concentration for substantially increased seeding densities in production was also proposed in this study. Following N-1 perfusion expansion, the concentration of the cell culture by using the same ATF system to remove the cell-free spent media could dramatically increase the end cell density achieved at N-1 stage by 2–4 folds, thereby affording to raise the production inoculation density considerably while keeping an appropriate split ratio. Due to the N-1 concentration, a higher seeding density of 20-80 × 10^6 cell/mL was attainable in production, easily exceeding the highest seeding density (~20 × 10^6 cell/mL) ever reported in IFB cultures. After being applied in different CHO cells producing different types of products, significantly improved productivities, up to ~6 folds of traditional fed-batch (TFB) and ~3 folds of IFB protein production, were obtained. Therefore, the UI-IPFB process provided an alternative intensified cell culture strategy for achieving high productivity from a fed-batch process. The new process strategy was proven effective in multiple CHO cell lines and product types demonstrating its potential for a wide range of industrial applications to biopharmaceutical manufacturing.

2 | MATERIAL AND METHODS

2.1 | CHO cell lines and media

Eight recombinant CHO cell lines were used in this study. Cell lines A/B/C/D/E producing different types of mAbs were constructed from WuXi Biologics' proprietary CHO-K1 with selective antibiotics. The host cell lines of Cell line F (Selexis SURE CHO-M, constructed under antibiotics selective pressure), Cell line G (Lonza CHOK1SV using knocked-out glutamine synthetase as the selection marker) and

TABLE 1 Comparison of different mammalian cell culture modes.

	TFB	IFB	IPFB	UI-IPFB	Hybrid perfusion fed-batch	CFB/IPC
N-1 modes	Batch	Perfusion	Perfusion	Perfusion+Concentration	Batch	Batch
N seeding density ($\times 10^6$ cell/mL)	0.3–1.5	5–15	5–15	20-80	0.3–1.5	0.3–1.5
Media exchange via perfusion	×	×	Intermittent in mid-to-late phases	Intermittent in mid-to-late phases	Continuous in initial phases	Continuous w/o bleeding
Bolus feeding	√	√	√ (nonperfusion days)	√ (nonperfusion days)	√ (after early-stage perfusion)	×
Cell retention	×	×	√	√	√	√
Production duration (days)	14–16	10–14	14–16	14–16	14–16	14–20
Harvest modes	Batch	Batch	Batch	Batch	Batch	Batch (CFB)/continuous (IPC)

Abbreviations: CFB, concentrated fed-batch; IFB, intensified fed-batch; IPC, intensified perfusion culture; IPFB, intermittent-perfusion fed-batch; TFB, traditional fed-batch; UI-IPFB, ultra-intensified intermittent-perfusion fed-batch.

Cell line H (Thermo Fisher Scientific CHO-S, constructed by using the methotrexate selection system), were stably transfected to express a bi-specific antibody, a mAb and a fusion protein, respectively. For culture media, the same basal and feed media selected for each cell line were used for the comparison of different cell culture processes in this study.

2.2 | N-1 perfusion seed culture and concentration

Lab-scale N-1 perfusion was conducted in 3 L glass bioreactors with a working volume of 2 L. An ATF2 hollow fiber filter (Repligen Corporation) with a 0.2 μm pore size was connected to the 3 L for perfusion culture. During cultivation, the culture was maintained at 36.5℃ and 40% of dissolved oxygen (DO) constantly. Continuous perfusion was carried out from Day 2 to Day 5 with two different perfusion rates: one vessel volume per day (VVD) from Day 2 to Day 4 and two VVDs from Day 4 to Day 5. The ATF circulation rate was set at 0.5 L/min during the entire perfusion culture. The N-1 perfusion for 250 L pilot production run was conducted in 50 L single-use bioreactor (Thermo Fisher Scientific Inc.) with a working volume of 40 L. A larger scale of ATF4 filter with 0.2 μm pore size (Repligen Corporation) was used for perfusion culture with the same perfusion rates to the 3 L N-1. The circulation rate of ATF4 was set at 4 L/min to keep a similar circulation/filtration ratio.

For the UI-IPFB process, the N-1 culture was concentrated before production inoculation. More specifically, using the same ATF cell retention device, the cell culture volume was reduced by ~50% within 2 ~ 3 h after the end of N-1 perfusion, which corresponded to a doubled end viable cell density. The concentration operation was carried out by increasing the ATF harvest flow rate to 12 VVD and in the meantime terminating or maintiaining the perfusion of fresh media. The pressures in the recirculation and the harvest lines were monitored with on-line pressure sensors (PendoTECH) to detect any indication of membrane fouling. Meanwhile, all the process controls were kept unchanged, including DO, pH, temperature and agitation. The required amount of concentrated culture was then transferred into the production vessel.

2.3 | Cell culture in production

For both IPFB and UI-IPFB processes, bench-scale production was all performed in 3 L glass bioreactors connected with an ATF2 hollow fiber, while the pilot-scale production used a 250 L single-use bioreactor (Thermo Fisher Scientific Inc.) and an ATF10 cell retention device. It is noted that the pore size of the hollow fiber filters used in all the productions was 50 kD to retain produced products in the bioreactor. The pH was controlled within the range of 6.7–7.2, and DO was controlled at 40%. Constant agitation was set at 250 rpm and 100 rpm for 3 and 250 L bioreactors, respectively. During production, the initial temperature was set as 36.5℃ and then shifted to 33℃ before the cell density reached the peak. The feeding

of the culture was performed on nonperfusion days in a bolus mode. The total feeding amount relative to the initial working volume was 16% for TFB, 24% for IFB, 30%–50% for both IPFB and UI-IPFB processes. The intermittent media exchange schedule was performed according to the specific study designs. Vessel volume each time (VVT) is used in this study to measure the perfusion volume relative to the culture volume in a single perfusion cycle lasted for a specific period of time. Typically, each time of intermittent perfusion took 24 h unless otherwise specified. All studies were performed in duplicates or triplicates, and only representative or averaged results were shown in the comparison.

2.4 | In-process testing, PQA assays, and spent media analysis

Bioreactor cultures were sampled daily. Viable cell density (VCD) and viability were monitored via the trypan blue dye exclusion method using Vi-Cell Analyzers (Beckman Coulter Inc.) equipped with the Vi-Cell XR software version 2.04. Lactate and IgG antibody concentrations were off-line monitored by Cedex Bio HT Analyzer (Roche Diagnostics GmbH). The titer of the produced fusion protein was measured by ultrahigh-performance liquid chromatography (UPLC) using an ACQUITY UPLC BEH300 C4 Column (Waters Corporation).

For product quality analysis, the harvested supernatant was purified by either a lab-scale Protein A capture step with MabSelect SuRe™ resin (GE Healthcare) for mAbs and bi-specific antibody, or by CaptureSelect Barry Affinity RUO (Thermo Fisher Scientific) for the expressed fusion protein by Cell line H. Subsequently, the high-performance size exclusion chromatography (SEC-HPLC) by ACQUITY HPLC (Waters Corporation) was used for size variant analysis. Capillary gel electrophoresis measured via LabChip® GXII Touch HT (Perkin Elmer Inc.) was applied for Caliper_Reduced & nonreduced purity testing. The method of imaged capillary electrophoresis (iCE) using the iCE3 system (ProteinSimple) was used for charge variant profiling. Lastly, N-Glycan species (e.g., G0, G1F, G2F, and Man5) were distinguished by UPLC using ACQUITY UPLC H-Class PLUS (Waters Corporation).

The spent media from Day 4, Day 5, Day 7, Day 8, Day 10, and Day 11 were collected and filtered with a 0.2 um filter for inhibitory metabolite analysis including indole-3-carboxylic acid, isovalerate, phenyllactate and 3-(4-hydroxyphenyl) lactate. This analysis was performed by LC-MS/MS (Shimadzu, Kyoto, Japan) equipped with two LC-30AD pumps, a SIL-30AC auto-sampler, a CTO-20AC column oven, a LCMS-8050 triple quadrupole (QQQ) mass spectrometer with an electrospray ionization (ESI) source. A 2.1 × 150 mm Shim-pack GIST PFPP (Shimadzu Global Laboratory Consumables, P/N 227-30858-07) with the particle size of 3 µm was used with the flow rate of 0.35 mL/min at 40℃. The mobile phase A was ultrapure water with 0.1% formic acid, and the mobile phase B was acetonitrile with 0.1% formic acid. The elution step was performed in a gradient manner. The mass spectrometry used Multiple Reaction Monitoring (MRM), with the condition as follows: spray voltage 3.0 kV, nebulizing gas flow 3 L/min, drying gas flow 10 L/min, heating gas 10 L/min and collision induced dissociation gas flow 270 Kpa. The relative level of each inhibitory metabolite was obtained by comparing with its standard material.

2.5 | COG analysis

The COG analysis of drug substance manufacturing was performed for different process modes including TFB, IFB and UI-IPFB. The COG calculation considered all operating costs mainly related to labors and facility occupancy in both upstream and downstream processing by using an internal cost assessment tool. Generally, as the processing complexity and duration increase, the operating costs will be adjusted accordingly. Other than operating costs, also considered in the calculation was the consumption of materials and consumables, which included the cost of upstream materials (single-use bioreactors, media, disposable mixing and storage bags, media filters, tubing and probes, and ATF columns) and downstream materials (buffers, mixing and storage bags, membranes for filtrations, and resins). The consumption was based on the assumption that the process is scaled up to a 2000 L single-use bioreactor with an equivalent final working volume in commercial manufacturing, in which resins are reusable up to a certain life cycle limit. In general, the consumption of media, buffers, bags, filters and resins are regarded as scale- or productivity-dependent, which ramps up proportionately to both process scales and titer outputs. For all other materials like probes and ATF columns, the costs were counted based on their actual usage. For the COG comparison in this study, the percentages of COG reduction were given using TFB as the control.

3 | RESULTS AND DISCUSSION

3.1 | IPFB cell culture performance compared to IFB

To demonstrate the performance advantages of IPFB over IFB, a CHO-K1 cell line expressing an IgG1 monoclonal antibody (Cell line A) was used. Figure 1b describes the recommended intermittent perfusion schedule in an IPFB process with the first perfusion cycle triggered after temperature shift (TS) or peak VCD is reached. Following the first exchange, additional blocks of perfusion with various perfusion rates and durations are optional to apply. In this study, beside the IFB control, two different intermittent perfusion designs were evaluated: one with three VVT (vessel volume each time) of media exchange on Day 5 for 24 h; the other one with two windows of media exchanges (three VVT for 24 h each) starting on Day 5 and Day 9 separately. It is noted that all the IPFB production cultures performed in the study used an ATF hollow fiber with a 50 kD pore size to hold the products within the bioreactor vessel, which enabled similar batch processing to IFB in downstream purification.

As in a routine IFB process, the N-1 perfusion expansion was carried out to reach a final VCD of 50×10^6 cells/mL (data not shown), after which three production bioreactors were inoculated with the same seeding density of 10×10^6 cells/mL. During production, the total feed ratio reached 34% of the initial working volume in the two IPFB conditions versus 24% in IFB. Due to a similar process condition in the early stage of production, all three conditions reached a comparable peak VCD of ~30×10^6 cell/mL on Day 4. However, the distinction of the two different processes appeared first in the IFB control, in which much faster cell death was observed as revealed from the viability dropping to ~50% on Day 13 (Figure 2a,b). As a consequence, the IFB production was early terminated. In comparison, the viability of the two IPFB cultures were maintained above 60% till Day 14. Over the trend of production, the

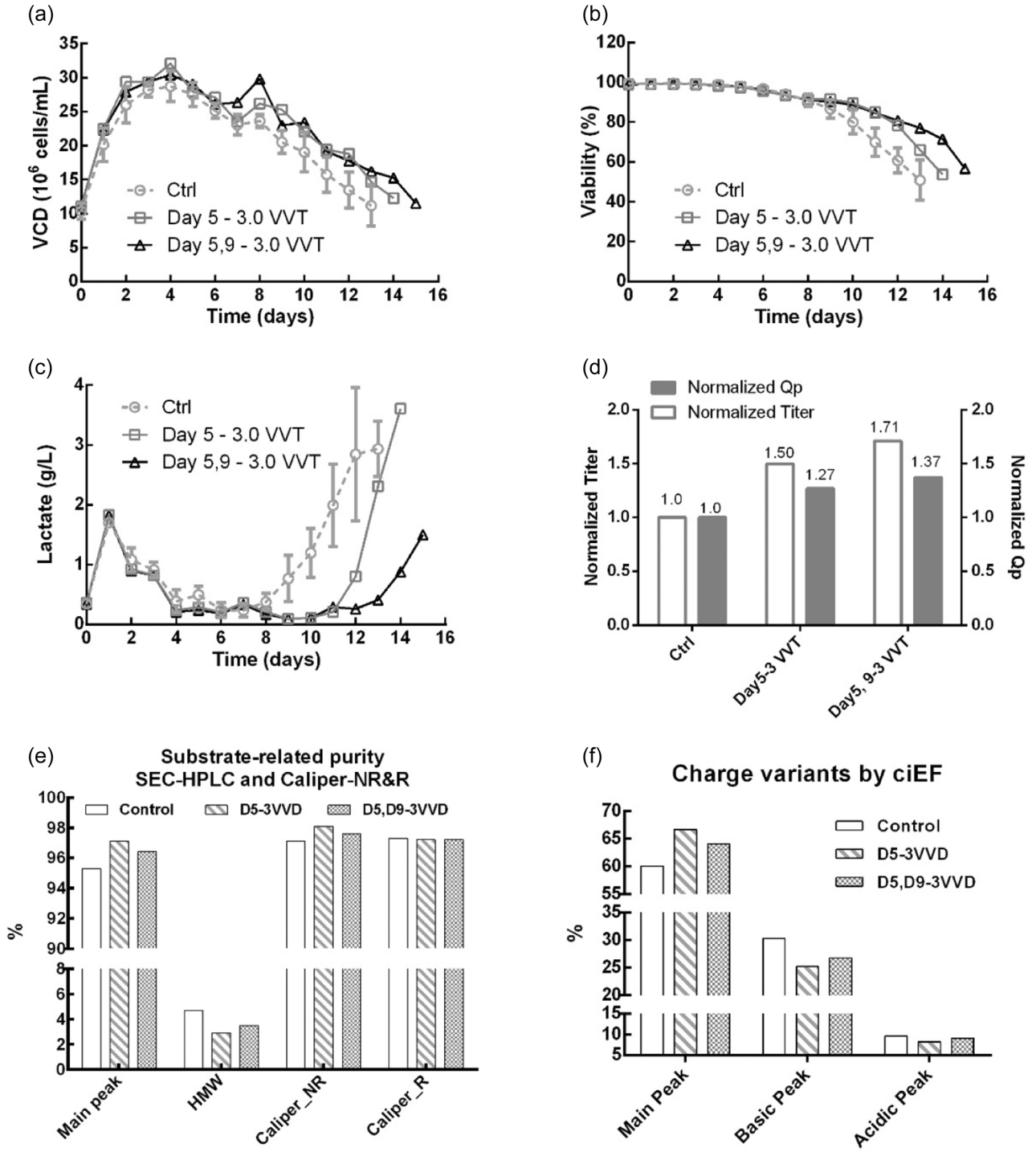

FIGURE 2 IPFB process performance with cell line A. (a) Viable cell density (VCD), (b) Viability, (c) Lactate metabolism, (d) Normalized Qp and Titer, (e) Product purity by high-performance size exclusion chromatography (SEC-HPLC) and Caliper NR&R, (f) Charge variant profile by cIEF.

two IPFB conditions outperformed the control by keeping a higher VCD and viability starting from Day 7. Concurrent to the VCD and viability drop, different levels of lactate resurgence were observed towards the end of the culture (Figure 2c), which implies a metabolic shift from the mitochondria-based tricarboxylic acid cycle and oxidative phosphorylation activities to cytosolic lactate reproduction, also known as Warburg effect (Hardie, 2022; Vaupel & Multhoff, 2021). In the IFB control, lactate started to rise sharply from Day 8 and reached ~3 g/L. In comparison, lactate reaccumulation was delayed by 4–5 days, and the extent of lactate resurgence was also reduced significantly especially after the second media exchange was applied in IPFB.

In terms of the final productivity, the earlier uptick in lactate as a reflection of metabolic disorder led to eroded productivity in the IFB control. The two IPFB conditions with one single or two separate media exchanges yielded ~50% and ~71% higher titers, respectively (Figure 2d). The higher titers were in line with the better VCD and viability maintenance and the postponed lactate upturn observed in the IPFB cultures. The advantages in final titer were also contributed by a lift in cell specific productivity (Qp) by 27%–37%. Besides, the two IPFB conditions also produced comparable or even better product qualities. Protein aggregation as seen from the HMW showed a reduction of ~2% in the products from IPFB cultures (Figure 2e). It was reported that cell apoptosis could have an unfavorable impact on both protein folding and processing capacity, leading to endoplasmic reticulum stress and unfolded protein response which would trigger even worse cell death (Fribley et al., 2009; Hetz et al., 2020). Besides aggregation, protein charge heterogeneity may affect product potency and stability (Ambrogelly et al., 2018; Du et al., 2012; Liu et al., 2009; Liu et al., 2014). Studies showed that both chemical and enzymatic modifications of proteins caused by the accumulation of toxic by-products, released intracellular enzymes and ROS may lead to a higher degree of charge heterogeneity (Chung et al., 2019; Miao et al., 2017; Vlasak & Ionescu, 2008). In this study, the two IPFB conditions refreshed culture media through intermittent perfusion and subdued the formation of charge variants as shown from the higher main peak and lower charge species (Figure 2f). As for N-glycan, some slight changes were noticed (~5% gap for G0F and ~3% for Man5), which suggested that minor N-glycan tuning may still be needed based on actual quality requirements (supplementary Table S1).

Many studies have reported the observations of irreversible and sometimes instantaneous cell death during a fed-batch process, which often led to low productivities (Ali et al., 2020; Horvat et al., 2020; Majors et al., 2008; Zhang et al., 2023). One possible reason is the suboptimal supply of nutrients such as amino acids, vitamins or growth factors in the bolus feeding paradigm of fed-batch cultures. As such, maintaining an optimized and balanced level of nutrients was critical for sustainable fed-batch performance. A previous study showed that the optimization of amino acid composition in media formulation could reduce lactate and ammonia levels, and increase the peak cell density by 55% and the titer by 27% (Xing et al., 2011). Ladiwala et al., also reported that the cell growth performance and productivity could be significantly improved through a rational amino acid feeding strategy to reduce inhibitory metabolites (Ladiwala et al., 2023). Another way to suppress cell apoptosis was through genetic engineering, such as the overexpression of negative apoptosis suppressors (BCL-2, BCL-xL, and MCL-1) and the knockout of positive inducers (BAK and BAX) (Cost et al., 2010; Zhang et al., 2018). Although these approaches were proven to be effective in some cases, in essence, none of them could fully resolve the underlying issue of deleterious by-product synthesis during the innate metabolic reactions and protein synthesis machinery. In this study, by performing intermittent perfusion in IPFB, it provided essential nutrients, and more importantly refreshed culture environment by removing or reducing toxic by-products. Through the analysis of the spent media, some inhibitors published previously, such as indole-3-carboxylic acid, isovalerate, phenyllactate and 3-(4-hydroxyphenyl) lactate (Mulukutla et al., 2017) was reduced markedly after each media exchange (Supporting Information Figure S2). The result indicated that IPFB could be used as a universal approach in alleviating the toxic effects from those commonly known metabolites such as lactate and ammonia, and those difficult or yet to be identified.

3.2 | Effects of intermittent perfusion rates and frequency

To further understand the effect of perfusion rates and intermittent perfusion cycles on the performance of IPFB, in this study we used the same Cell line A to explore different perfusion strategies in an IPFB process. A total of three IPFB conditions was studied: the first two involved the use of two lower perfusion rates (1 and 2 VVT) with the same starting timepoint and duration (24 h starting from Day 5); the third one extended the perfusion cycles to three times and each cycle exchanged a media volume of 1 VVT.

The three IPFB conditions observed a similar peak VCD of ~28×10^6 cells/mL and end viability of ~65% (Figure 3a,b). Similar to the conclusion from the previous study, IPFB with even 1 VVT of media exchange led to a higher viability towards the end of the culture from Day 8 to 14. Furthermore, the increased total media exchange rate in three separate cycles showed the most delayed lactate spike and the lowest lactate level (1.5 g/L vs. 3 g/L in the control) (Figure 3c). As the number of cycles and the volumes of media exchange increased from 1 VVT to a total of 3 VVT, the final normalized titers climbed up to 1.52-fold of the control (Figure 3d). Interestingly, the two IPFB processes which underwent the same total perfusion volume but with two different perfusion schedules (1 VVT on Day 4, 7, and 10) (Figure 3d) versus 3 VVT on Day 5 (Figure 2d) exhibited the same final normalized productivity (~1.5-fold), only slightly higher than the one with 1 or 2 VVT on Day 5 (1.30- and 1.43-fold, respectively). The observation implied that by performing the first media exchange with 1–2 VVT on Day 5 contributed to most of the titer improvement, and additional cycles of perfusion could boost

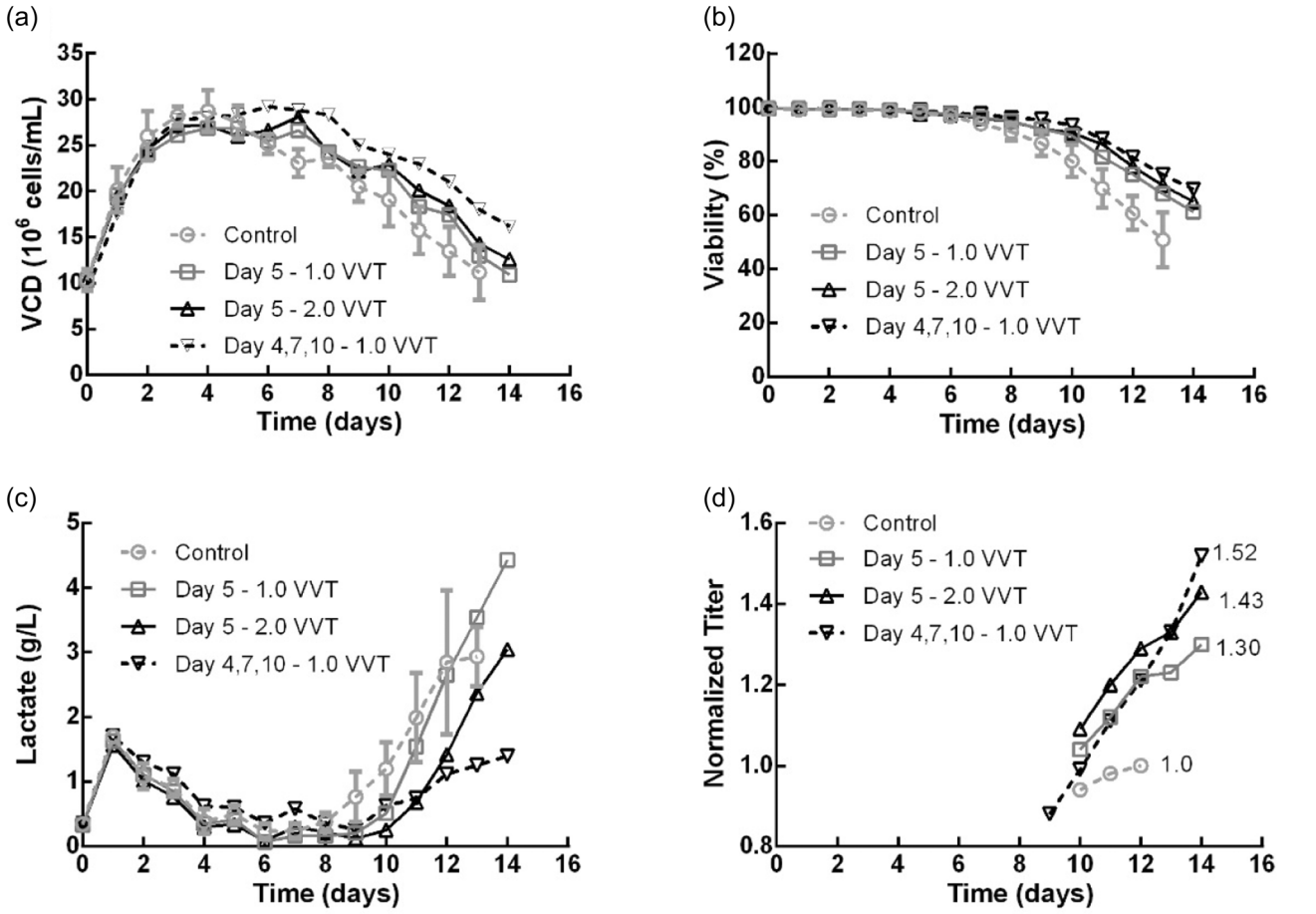

FIGURE 3 Effects of intermittent perfusion rates and frequency on the performance of IPFB. (a) Viable cell density (VCD), (b) Viability, (c) Lactate metabolism, (d) Normalized Titer.

productivity even more. This may be due to fact that initializing the first one when the cells entered the stationary stage and before viability began to descend contributed to the early removal of inhibitors, leading to a more effective process to counteract the evolving cell apoptosis which often came with irreversible metabolic shift. However, if the intention was to further ensure better cell status towards the end of the production, the condition with the three separate perfusion cycles ended up with higher harvest viability (70% vs. 60%) and lower end lactate (1.5 g/L vs. 3.5 g/L), compared to the one with 3 VVT on Day 5 (Figure 2b,c). In addition, more diverse intermittent perfusion schemes, including the durations, intervals and exchanged volumes of each perfusion cycle, were evaluated (Supporting Information Figure S1). All the conditions exhibited a similarly lessened decreasing trend in VCD and viability, suppressed lactate uptick and ultimately improved titers compared to the control, which suggested that the operation agility of IPFB cultures as seen from the relatively flexible perfusion schedules and optimized perfusion volumes could simplify manufacturing operations especially in the preparation of perfusion media and ATF replacement in case of filter fouling.

3.3 | UI-IPFB cell culture performance compared to IFB and IPFB

Due to the limited cell proliferation potential in N-1 perfusion, the seeding densities of the IFB or IPFB process in the prior case studies were all around 10×10^6 cell/mL, which was determined by keeping an appropriate split ratio based on the final cell density (~50×10^6 cell/mL) at the end of N-1 perfusion. To maximize the intensification potential, this study proposed an ultra-intensified fed-batch strategy (UI-IPFB) with the goal to considerably elevate production seeding density by applying a cell culture concentration step using the same in-use ATF perfusion device at the end of N-1 perfusion cultivation. To demonstrate the ultra-intensified strategy, a CHO-K1 cell line (Cell line B) expressing an IgG1 mAb was used. The N-1 peak VCD of Cell line B reached ~50×10^6 cell/mL after 3-day perfusion and was then quickly concentrated to ~100×10^6 cell/mL within 2–3 h via the ATF filtration (Figure 4a). With the concentrated culture, the production seeding density was doubled to ~20×10^6 cell/mL with a similar split ratio to the IFB and IPFB conditions. The UI-IPFB production followed a perfusion schedule of 1 VVT on Day 4, 7, and 10.

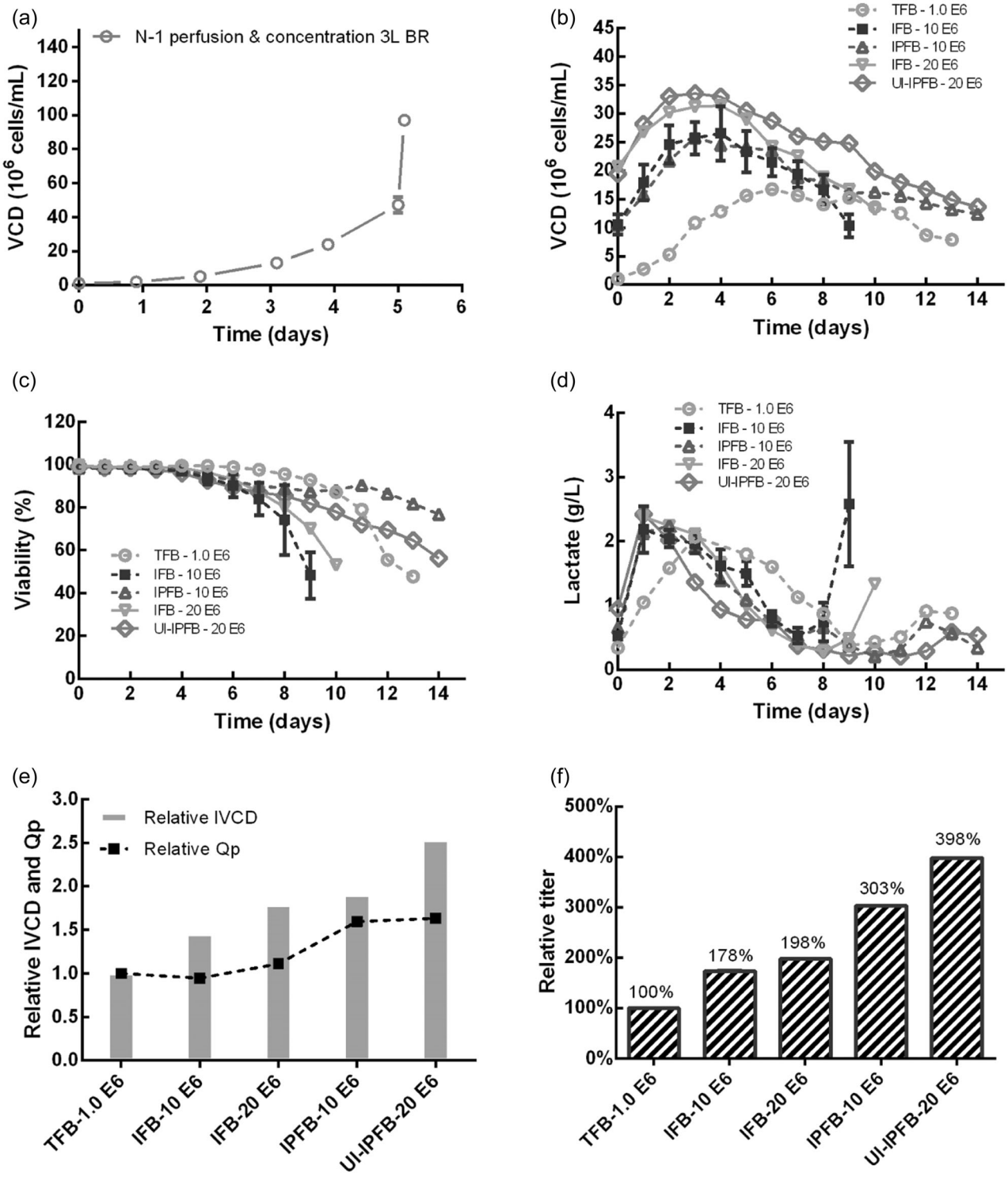

FIGURE 4 Ultra-intensified intermittent-perfusion fed-batch (UI-IPFB) cell culture performance compared to intensified fed-batch (IFB) and intermittent-perfusion fed-batch (IPFB). (a) Viable cell density (VCD) of N-1 stage, (b) Production VCD, (c) Viability, (d) Lactate, (e) The cumulative integral of viable cell density (IVCD) and Qp, (f) Relative titer results.

Similar to the IPFB performance seeded at 10×10^6 cell/mL, the UI-IPFB culture seeded at 20×10^6 cell/mL showed improved performance on VCD and viability maintenance until Day 14, while the IFB processes seeded at either 10×10^6 cell/mL or 20×10^6 cell/mL ended up with early termination due to viability crush to ~50% (Figure 4b,c). The initiation of cell crush was almost concurrent to the lactate spike on Day 8 and 9 (Figure 4d). Given the sharp decline in cellular performance in the two IFB conditions, only marginal difference in Qp was found compared to the TFB seeded with a regular cell density of 1×10^6 cell/mL.

Owing to the joint benefit from the ultrahigh seeding and the IPFB culture mode, the cumulative integral viable cell density (IVCD) of UI-IPFB rose by 40%–70%, compared to the two IFB controls. Moreover, the surge in the lactate was also suppressed effectively with the IPFB strategy (Figure 4d). The Qp from UI-IPFB increased by ~70% compared to the IFB controls (Figure 4e). Overall, the final titer reached ~4-folds of the TFB and ~2-folds of the two IFB controls (Figure 4f).

Typically, the initial production seeding density is largely determined by the proliferation potential of cells in N-1 perfusion affected by a variety of factors including cell doubling time, perfusion medium strength, and the maximum cell density that a stirred tank bioreactor can support without inducing undesirable cell death due to rising oxygen transfer demand and shear stress from intensive aeration (Ben Yahia et al., 2023; Woodgate, 2018). Therefore, it has long been a major bottleneck in developing an adequate N-1 perfusion process to realize high density seeding in fed-batch production. In this study, the idea of cell culture concentration via the ATF system provides an effective solution for intensified cell expansion through perfusion culture. Our result also suggested that as high as 6.8 times of concentration could be achieved with a final cell density reaching ~410×10^6 cell/mL with no sign of ATF membrane fouling, with which a much higher seeding density of >80×10^6 cell/mL was achievable in a production culture (Supporting Information Figure S3). It is also worth noting that this method could be readily implemented in manufacturing without any major modification on the existing facility or equipment.

The UI-IPFB process provided an ultra-intensified fed-batch strategy with the final productivity competitive to continuous processes such as CFB. Using the same Cell line B, the final titer from the CFB production (data not shown) was on a par with the one from the UI-IPFB process. However, the CFB production endured more complex operations and consumed three times more media during the continuous perfusion, leading to uneconomical operation and media cost. Besides, CFB requires more footprint for large-scale media usage and disposal and more back-up ATF filters, while UI-IPFB can greatly simplify manufacturing operations and ease the facility burden of repeated media preparations and ATF changeovers.

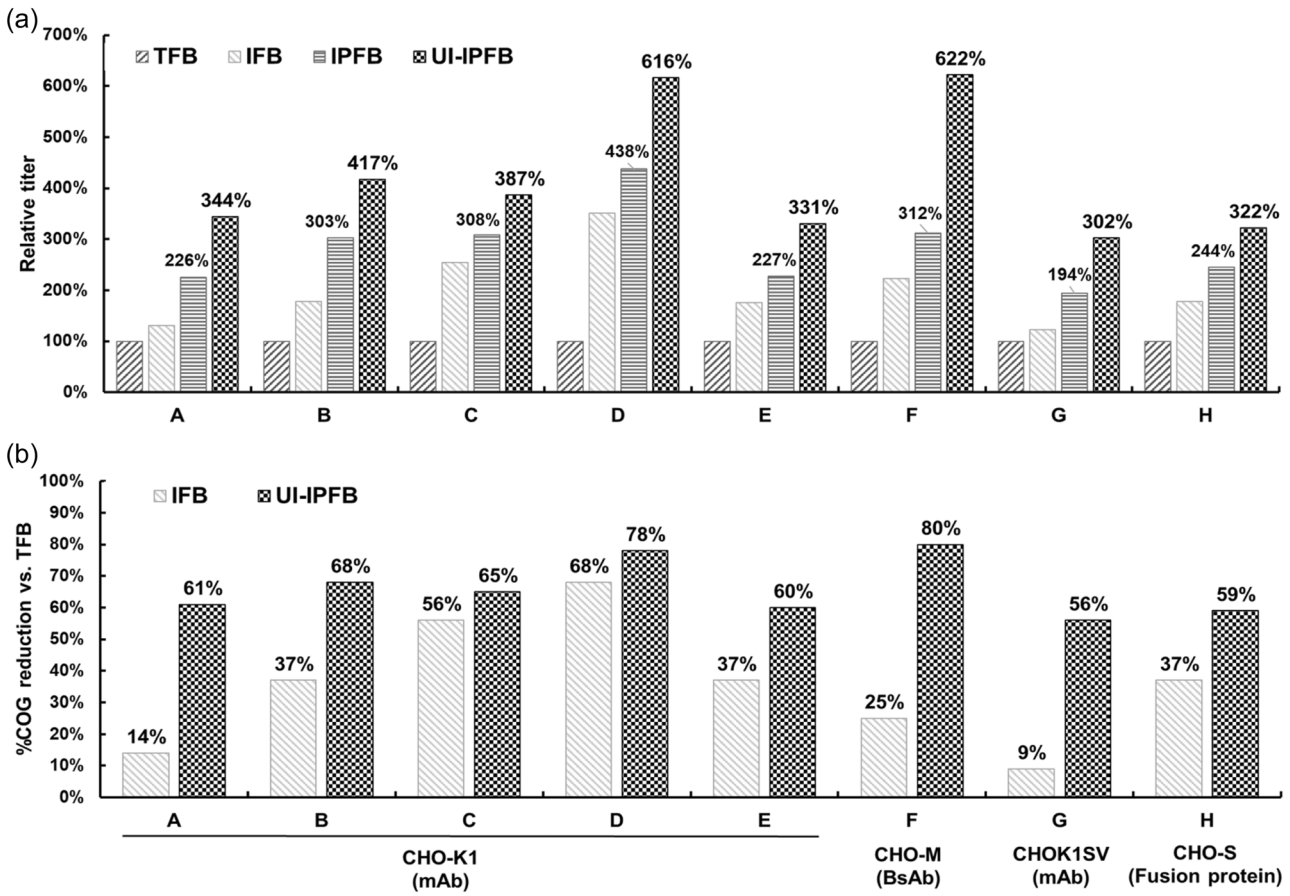

FIGURE 5 Productivity comparison of traditional fed-batch (TFB), intensified fed-batch (IFB), intensified intermittent-perfusion fed-batch (IPFB) and ultra-intensified intermittent-perfusion fed-batch (UI-IPFB) in different cell lines expressing different types of molecules (a), and the corresponding percentages of cost of goods (COG) reduction after converting from TFB to IFB or UI-IPFB, assuming at a 2000 L single-use manufacturing scale (b). COG calculation based on the expenses from consumables, materials, equipment, and other operating costs from labors and facility usage.

3.4 | Performance of IPFB and UI-IPFB in different cell lines

To demonstrate the performance of IPFB and UI-IPFB on different cell lines expressing various molecular types such as mAbs, bispecific antibodies or fusion proteins, a total of eight CHO cell lines (including Cell line A and B) were cultured using the IPFB or UI-IPFB strategy. A summary of the normalized titer results was shown in Figure 5a. As expected, the IFB process recorded only modest productivity improvement by an average of ~100% compared to the TFB controls. Notably, some cell lines, including Cell line A and G, underperformed particularly in the IFB mode, which showed only minimal titer changes. As for IPFB, the final titer grew by an average of ~170% from the one of TFB. Even for Cell line A and G that appeared to be unfit for the IFB process, the use of the IPFB strategy could lead to a titer increase by up to ~100%. After applying the UI-IPFB process, the titer performance stood out in all the cell lines studied, climbing to 3–6 folds of the TFB controls. Beside productivity improvement, it is also worth mentioning that comparable product quality profiles were observed in the different molecules expressed with only minor differences existed for some of the products (data not shown). The result indicated that both IPFB and UI-IPFB could be used as universal approaches for process intensification on a host of CHO cell lines as well as products of interest. To further demonstrate scalability, the UI-IPFB process of Cell line B was scaled up from 3 L bench scale to 250 L single-use bioreactor (SUB) using a 50 L SUB for N-1 ATF perfusion and concentration, which exhibited highly comparable in-process trends in both N-1 and N stages (Supporting Information Figure S4). The highly comparable performance was evidence of the process scalability in large-scale manufacturing.

Compared with other representative studies related to process intensification (Table 2), UI-IPFB exhibited its unique features in resolving

TABLE 2 Summary of representative studies using different intensified processes.

Intensified process	Cell line	Molecule type	Harvest titer (g/L)	% Titer increase versus TFB	Key findings	References
IFB	CHO-K1	mAb	NA	Comparable	Achieved comparable titer within a shorter culture duration of 8 days.	Padawer et al., (2013)
	CHO-DG44	IgG1	4.5	90%	High seeding and bolus lactate feeding increased titer by 90% in equivalent runtime; Transcriptome analysis revealed key regulators associated to culture phase progression.	Stepper et al., (2020)
	CHO-K1 GS	mAbs	NA	~200%	Increased seeding density to 10–20 × 10^6 cell/mL with redesigned media and optimized temperature shift strategy.	Xu et al., (2020a)
	CHO-DG44	IgG1	~3.5	Comparable	Added butyric acid to increase Qp; harvested on as early as Day 7 due to rapid viability drop.	Schulze et al., (2022)
	CHO-K1 GS	mAb	NA	300%/700%	Higher seeding densities showed not only higher IVCD but also higher Qp; IFB were seeded from enriched or perfusion N-1 expansion.	Xu et al., (2020b)
CFB	CHO	mAbs	As high as 25.3	~200%	Intensified production output with no change in volumetric capacity; titer improvement was mainly due to increased IVCD.	Yang et al., (2016)
	CHO	IgG4	~20	~700%	Titer increase proportional to the increase in IVCD; nearly 6-fold higher HCP level than that of TFB.	Lu et al., (2023)
	CHO-GS	mAb	21.4/36.7	300%/580%	Enriched perfusion media contributed to significant improvement in Qp and IVCD.	Xu et al., (2017)
	CHO-GS	IgG1	NA	476%	Copper addition improved lactate metabolism in an intensified upstream process.	Xu et al., (2016)
WuXiUP^{TM} (IPC)	CHO	Fc-fusion protein	52.14	1749%	Both IVCD and Qp were significantly improved by continuous perfusion; Continuous product purification needed for downstream processing.	Zhou et al., (2021)

the common bottlenecks in an IFB culture and delivering a desirable process performance. Xu et al reported 4-fold and 8-fold increase in titer by using enriched or perfusion seed expansion, respectively, in which an even better viability was obtained in the IFB processes compared to the TFB control (Xu et al., 2020b). It is likely that such a successful IFB case could be further intensified using the UI-IPFB strategy in which both IVCD and cellular productivity could be maximized even more. However, the development of an IFB process may not be as straightforward as it appeared to be. As reported by Schulze et al, the increase in production seeding density failed to improve the final volumetric productivity, even though considerable enhancement in Qp was observed after butyric acid addition (Schulze et al., 2022). Similarly, the worsening cell performance during the progression of the IFB cultures was also identified to be the main cause of poor productivities. Under the circumstance, the development of IPFB and UI-IPFB processes aimed to overcome the declining cell performance and maximize the potential of fed-batch based intensification for better productivities. On the other hand, continuous processes including CFB and IPC were also extensively studied and applied previously in process development. Xu et al., gave a deep analysis on the different intensified cell culture processes including TFB, IFB and CFB (Xu et al., 2017). Although CFB presented the highest supernatant productivity, the authors pointed out that the media cost per unit product was nearly doubled in the CFB mode, compared to the IFB process. As a result, the CFB process was proven to be suboptimal in the final digestion of manufacturing COG. In this study, UI-IPFB proposed a novel upstream strategy leading to more efficient and balanced use of media, thereby reducing media consumption and costs compared to continuous cultures. During manufacturing, even if UI-IPFB involves additional equipment and media costs from the designed perfusion operation in both N-1 and production, and higher consumption of downstream purification consumables and materials due to an increased amount of harvest products, and other related operating costs such as labors and facility occupancy, the comparative advantage of UI-IPFB, which is displayed in the much higher productivity than other fed-batch or perfusion processes, contributes to a considerable reduction in manufacturing COG, a calculated ratio of total manufacturing costs to the purified protein yielded per batch. Based on the COG calculation including raw materials, equipment, labors and facilities, the conversion from TFB to IFB resulted in limited COG decrease by 9%–37% for six of the eight cell lines and by 56%–68% in only two cell lines studied (Figure 5b). In contrast, UI-IPFB could drive down the overall manufacturing COG by at least 60%, and as high as 80% COG reduction was achievable in Cell line D and F, which produced up to 6 times of products per batch compared to TFB. As demonstrated, UI-IPFB is proven to be an effective process strategy for cost-effective manufacturing of biologics.

4 | CONCLUSION

Process intensification with the objective to improve productivity plays a critical role in the development life cycle of biopharmaceuticals. Such efforts set the stage for product commercialization and ultimately exert an impact on patient access. To provide an alternative solution for intensification, we developed two novel cell culture modes on the basis of the current fed-batch and perfusion culture practices. One is the IPFB process which is characterized by intermittent perfusion through the ATF system in IFB production, which exhibiting better cell performance, enhanced Qp and volumetric productivity compared to IFB. The other one is UI-IPFB which is realized by cell culture concentration after N-1 perfusion expansion to obtain a much higher seeding density in the IPFB production process. For the eight cell lines studied, it was shown that the IPFB and UI-IPFB processes could achieve an average of 2.8 and 4.2 folds of TFB productivities, respectively. In terms of large-scale manufacturing, IPFB and UI-IPFB inherit both the operation simplicity of fed-batch cultures and the advantage of superior cell sustainability in a perfusion process. Lastly, UI-IPFB could also offer more competitive manufacturing COG with greatly increased protein production.

AUTHOR CONTRIBUTIONS

Shaoxun Xiang, Jinliang Zhang, Le Yu and Jun Tian: Conceptualization; experimental design and planning. **Shaoxun Xiang and Le Yu**: Manuscript writing. **Wenxiu Tang, Hao Tang, Kecui Xu, Xin Wang, Yanyan Cui and Kaidi Ren:** Study execution; data collection and analysis. **Weijia Cao and Yuning Su**: Review of data analysis and manuscript. **Le Yu, Jun Tian and Weichang Zhou**: Supervision, resources and review.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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