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Stepwise cell culture process intensification for high-productivity and cost-effective commercial manufacturing of a Mabcalin™ bispecifics

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ABSTRACT

Process intensification and media optimization, as a crucial step for improving productivity and manufacturing cost of goods (COG), set the stage for commercialization readiness and redefine the landscape for patient access. This study described a stepwise approach to explore different intensified fed-batch processes along with optimized cell culture media for the production of a Mabcalin™ bispecifics. Initially, by leveraging perfusion expansion, intensified fed-batch (IFB) with an inoculation density of 10.3×10^6 cells/mL was developed to produce 6.1 g/L of products, compared to 3.9 g/L from the original traditional fed-batch (TFB). Following the IFB conversion, a high-performing production medium, MagniCHO™, was chosen to substitute the original one, which further boosted the titer to 9.1 g/L. The result underscored the significance of developing an optimized cell culture media for intensified cultivation. Furthermore, the approach of ultra-intensified intermittent-perfusion fed-batch was utilized, raising the seeding density to 73.6×10^6 cells/mL. A final harvest titer of 24.5 g/L was recorded. Additionally, manufacturing COG was calculated to evaluate how process intensification could lead to improved manufacturing cost-effectiveness, with up to 71 % COG reduction attainable with the UI-IPFB process. This study demonstrated that even for difficult-to-express modalities, applying a strategic development approach including process intensification and media optimization could effectively improve manufacturing efficiency and COG competitiveness.

1. Introduction

Bispecific therapeutics, engineered to possess two specific antigen-binding sites, are emerging as a highly promising therapeutic modality due to their potential for more effective clinical therapeutic responses by targeting oncogenic signaling pathways and cytokines [1,2]. Since the FDA approval of blinatumomab (Blincyto; Amgen) as the first bispecific antibody (BsAb) in 2014, a total of seven BsAb molecules have been approved in the United States and Europe to date [3]. Despite high market expectations, the complex bispecific structure often presents many technical challenges in molecule design and manufacturing process development, making the high-productivity manufacturing of bispecific products difficult [4,5]. Unlike monoclonal antibodies (mAbs), BsAbs often require the co-expression of three or more distinct genes targeting different antigens, followed by their assembly into a specific format, placing significant stresses in protein expression and folding machinery [6]. As a result, the complex architecture of BsAbs often results in lower expression levels owing to reduced folding and secretion efficiency, compared to monoclonal antibodies. This is further exacerbated by the tendency for mispairing of heavy and light chains during the assembly process of asymmetrical BsAbs, which results in the accumulation of non-functional or misassembled molecules subject to removal during downstream processing [7]. Additionally, BsAbs are susceptible to physical and chemical degradation, which may yield high levels of aggregation, clipping, and mispairing during cell culture production [8]. In consequence, optimizing cell culture conditions, media composition, and feed strategies were among the common approaches employed to enhance the yields of BsAb production[9,10]. Otherwise, a low-yield production could necessitate hefty capital investment on large-scale manufacturing facilities and result in alarmingly high cost of

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goods (COG), creating a significant barrier to market supply and affordability.

In recent years, to address the growing demand for more affordable biotherapeutics, significant technological advancements have been made. Among these, cell culture process intensification continues to draw increasing attentions [11–13]. Many of these intensification strategies employed intensified seed expansion methods, such as perfusion or enriched seed cultivation, aiming to obtain a higher initial inoculation density at the production stage. As reported in prior studies, the so-called IFB culture mode offers two key benefits: it increases daily or volumetric productivity by reducing the duration of cell lag and proliferation phases, and in turn, it also leads to shorter batch processing times, maximized facility capacity and efficiency [12,14]. Schulze et al. observed a 36 % increase in space-time yield under an IFB production with a seeding density of 5.0×10^6 cells/mL, even though the final volumetric productivity remained similar [15]. Similarly, Yang et al. noted that switching to IFB production could increase manufacturing capacity by 30 % due to the reduced duration of IFB cultures [16]. Additionally, Xu et al. developed an intensified seed culture scheme, either through enriching the N-1 seed culture medium or by operating the N-1 step in a perfusion mode, recording a harvest titer increase by 4–8 folds within a runtime equivalent to traditional fed-batch [17]. However, often encountered in the development of a high-density IFB process is the challenge from the ever-increasing accumulation of metabolic by-products, which are often toxic inhibitors produced from inefficient and poorly regulated metabolic reactions [18,19]. Studies reported that the uptrend of lactate, ammonia, and a set of reactive oxygen species during an intensified process was a primary cause of suboptimal process performance [20,21]. It emphasizes the importance of optimizing production culture conditions to minimize unfavorable metabolic limitations when practicing intensified bioprocessing strategies [22,23].

Alternatively, Intermittent-Perfusion Fed-Batch (IPFB) was previously demonstrated as an effective approach to resolve the potential IFB issues [24]. Under a high-cell-density condition often involving undesirable cell metabolism in the mid- to late-stage of production, employing intermittent medium exchanges via perfusion was capable of replenishing nutrients and removing inhibitory metabolites. Furthermore, an ultra-intensified IPFB (UI-IPFB or WuXiUI™) was reported to increase titers by up to six folds, compared to an TFB process. It applied a continuous perfused and then concentrated N-1 seed culture to greatly increase seeding densities, in addition to the intermittent perfusion operation during production [24].

Equally important for process intensification is the development of a customized and “fit-for-process” media formulation, which has long been used as an effective way to enhance cell culture performance and productivity [25,26]. The components within a chemically defined medium are complex and, in most cases, need to be optimized specifically for different cell lines. Consequently, cell line-specific medium screening and optimization, employing either empirical or rational approaches, have been extensively utilized for troubleshooting or achieving a specific objective [27]. Through targeted optimization and the implementation of dynamic feeding strategies, significant improvements in production titers were reported: from 5.0 to 6.3 g/L with cell line A, and from 4.0 to 9.0 g/L with cell line B, as highlighted in a previous study [28]. Yongky et al. demonstrated that a well-fortified medium was crucial for the N-1 seed culture to achieve sufficient cell biomass proliferation with sustainable viability for the following IFB inoculation and production [29]. Furthermore, Schulze et al. found that supplementing cell culture media with butyric acid could increase cell-specific productivity from 25.0 to 37.0 pg/cell/day in an IFB process [15], although the beneficial effect of butyric acid was also reported be cell line specific [30]. The conventional and prevailed approach for media formulation development mainly included empirical component screening, rational experiment designs and extensive wet-lab studies, with the purpose to explore and confirm the optimal ranges of essential nutrients, such as amino acids, vitamins, minerals, and trace elements [31,32]. More recently, the application of metabolic modeling multivariate analysis in cell culture media design offered advanced insights into cell culture behavior at both the statistical and mechanistic levels [33,34]. Therefore, a variety of sophisticated approaches through either data-driven designs or model-based simulations have been published for media optimization to achieve improved productivity and product quality.

Leveraging existing knowledge and process strategies for high-productivity and cost-effective manufacturing processes, this study outlined the development path for Pieris Pharmaceutical, Inc.’s Cinrebafusp Alfa (PRS-343), a bispecific fusion protein that combines 4-1BB-targeting Anticalin® proteins and a HER2-targeting antibody (Fig. 1). This novel class of bispecifics with single-chain design is henceforth referred to as Mabcalin™ proteins [35]. The study applied diverse process strategies, including TFB, IFB, and UI-IPFB (Fig. 2), with different media options. To ensure optimal cell growth, viability, and titer in intensified upstream processes, WuXi Biologics, Inc. developed a proprietary production medium, MagniCHO™, tailored for intensified cultivation. The development was grounded in a comprehensive understanding and simulation of key nutrient requirement of various CHO cell types under different metabolic stages. Switching to MagniCHO™ from the original commercial media showcased the combined effect of process intensification and media optimization. Our strategy using the UI-IPFB process, a hybrid of fed-batch and perfusion cultivation, fundamentally transformed the process performance, achieving an unprecedented titer of 24.5 g/L from the original titer of 3.9 g/L. This titer is over fivefold higher than that of the original TFB process, while maintaining comparable product quality. Additionally, the study provided a thorough COG breakdown analysis under different manufacturing process conditions, elucidating the effect of process intensification on cost distribution and COG reduction. As a result, up to 46 % and 71 % reduction in COG could be realized through the IFB and UI-IPFB process development, respectively. Overall, this study demonstrated a systematic and effective approach to developing a high-productivity process for a complex bispecific protein, highlighting the decisive role of process intensification and media optimization in changing the efficiency and cost-effectiveness of biotherapeutic manufacturing.

2. Materials and methods

2.1. Cell line, media, and seed train

The parental cell line used in this study was the Selexis SURE CHO-M Cell Line™, stably expressing Pieris Pharmaceutical, Inc.’s cinrebafusp alfa (PRS-343), a bispecific fusion protein that combines 4-1BB-targeting Anticalin® proteins genetically linked to a HER2-targeting

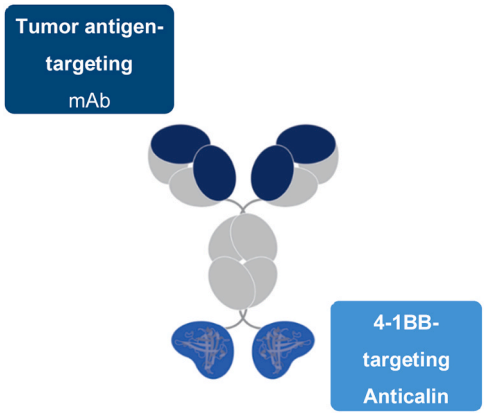

Fig. 1. Architecture of Pieris’ proprietary Mabcalin™ bispecific proteins.

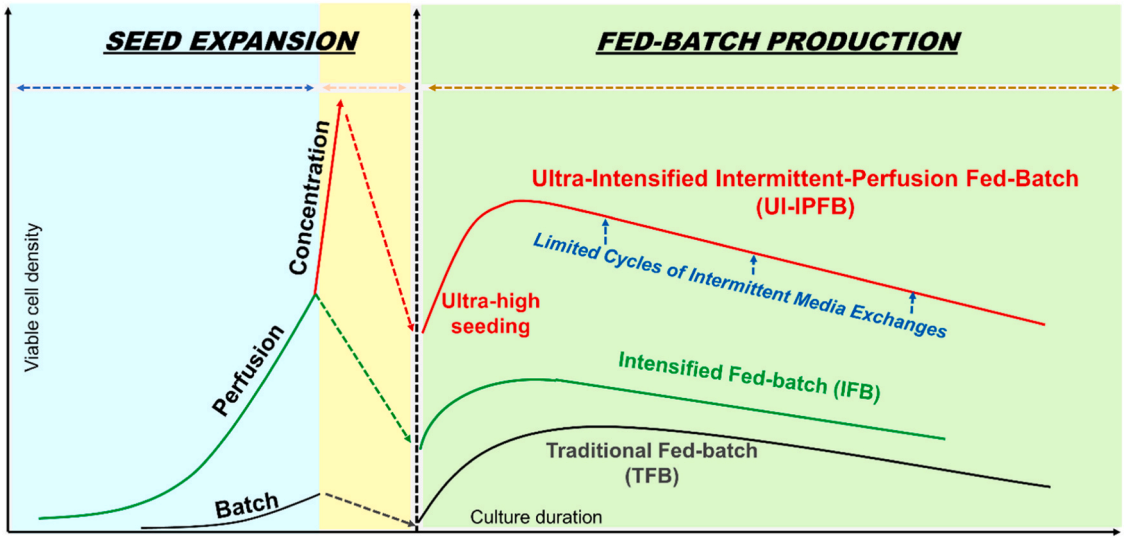

Fig. 2. Schematics of Ultra-Intensified Intermittent-Perfusion Fed-Batch (UI-IPFB) process in comparison to Traditional Fed-Batch (TFB) and Intensified Fed-Batch (IFB).

antibody. Four commercially available and chemically defined cell culture media were evaluated in the study, which included Media A (BalanCD CHO Growth A from FUJIFILM Irvine Scientific), Media B (Actipro from Cytiva), Media C (Ex-CELL® Advanced CHO from Merck SAFC), Media D (Dynamis from Thermo Fisher). A proprietary production medium MagniCHO™™ was developed by WuXi Biologics, Inc., as one of the platform media used in multiple CHO cell culture processes. The same set of commercial feed media HyClone™ Cell Boost™ 7a and 7b (Cytiva) was used in all the conditions.

During the cell expansion stage, cells were cultured in shake flasks under the conditions of 36.5 ℃, 125 rpm rotation, and 6 % CO_2 in a shaking incubator. The shake flasks were seeded at 0.3 × 10^6 cells/mL and were sub-cultured every 3–4 days when a VCD range of 3.0–6.0 × 10^6 cells/mL was reached.

2.2. N-1 seed cultures

The N-1 seed culture were performed in shake flask or 3 L bench-scale bioreactors in either batch or perfusion mode. Similar to the cell expansion stage, the culture temperature was maintained at 36.5 °C. The parameters for the shaking incubator with an orbital diameter of 50 mm were 6 % CO2, 80 % humidity, and 125 rpm rotation. In the perfusion culture of 3 L bioreactors, the percentage of dissolved oxygen (DO) was maintained at 40 % by air or oxygen sparging, and the pH was controlled at 6.90 ± 0.25 using pure CO_2 sparging or 1 M sodium carbonate solution titration. Antifoam was supplemented as needed to control foaming in the bioreactors.

For N-1 batch expansion, the culture was seeded at 0.3 × 10^6 cells/mL and lasted for 3 days until the VCD reached 3.0–6.0 × 10^6 cells/mL. For N-1 perfusion expansion, the culture was seeded at 1.0 × 10^6 cells/mL. An ATF-2 hollow fiber filter (Repligen) was connected to the bioreactor for continuous perfusion from day 2 to day 5. The perfusion speed was ramped up daily with a three-day perfusion rate of 1.0, 2.0 and 3.0 VVD for Media A as the perfusion medium, and 0.5, 1.0 and 3.0 VVD when MagniCHO™ was used. For N-1 perfusion in the UI-IPFB study, the culture seeding density and perfusion rates were maintained the same. Prior to UI-IPFB production inoculation, the N-1 seed was concentrated to 50 %, 33 %, 25 %, 20 % of the initial culture volume within 4 h by using the in-use ATF-2 cell retention device. During the cell culture concentration stage, the bioreactor parameters, such as temperature, pH, DO, agitation, and gassing, were maintained under the similar settings. The same perfusion rate of 3.0 VVD was applied during concentration, while the ATF harvest flow rate was increased to 9.0 VVD to quickly concentrate the culture volume.

2.3. Production cultures

Four commercially available media (A–D) were evaluated in TFB and IFB modes using 250 mL shake flasks. The fed-batch production for media screening was performed for 14 days or terminated if viability declined below 60 %. The target inoculation density of TFB and IFB production was 1.0 × 10^6 cells/mL and 10.0 × 10^6 cells/mL, respectively, unless otherwise specified for individual design. The initial culture temperature was 36.5 ℃ and then shifted to 33.0 ℃ on the day before the peak VCD was reached.

The production bioreactor with Media A and MagniCHO™ was carried out in 3 L bioreactors. The same commercial feed media Cell Boost 7a and 7b were fed daily in a 10:1 ratio. Due to the varying seeding densities and metabolic consumption in the different process conditions, the feeds were supplemented based on the daily consumption as indicated by the off-line measured osmolality. As a result of daily feeding, osmolality was maintained between 350 and 450 mOsm/kg. Overall, both of the TFB and IFB production entailed a total feeding ratio of 33–36 %, while the UI-IPFB production required a total feeding amount of ~ 80 % of the initial working volume. Dissolved oxygen was maintained at 40 % by air or oxygen sparging, and the pH was controlled between 6.9 and 7.0 using pure CO_2 sparging or 1 M sodium carbonate solution titration. Antifoam was supplemented as needed to control foaming in the bioreactors. Temperature was set as 36.5 ℃ initially and then shifted to 33.0 ℃ or 32.0℃ one day before the peak VCD was reached. The initial inoculation density of TFB and CFB production was 0.3 × 10^6 cells/, while IFB was seeded at 10.0 × 10^6 cells/mL. For the UI-IPFB production, the target inoculation densities were 25.0, 40.0, 55.0 and 80.0 × 10^6 cells/mL.

The intermittent or continuous perfusion strategy was performed according to the specific study designs. For the UI-IPFB process, three separate intermittent perfusion cycles were performed on day 4, 7 and 10, with each cycle having a perfusion rate of 1.5 VVD for 24 h. For the CFB process, continuous perfusion started on day 2 with a perfusion rate of 0.5 VVD, and then ramped up to 1.0 VVD from day 3 onwards. In all the production bioreactors, the protein was retained in the bioreactor vessel during perfusion by using a hollow fiber pore size of 50 kD, as described in the previous study [24].

2.4. In-process testing and quality attribute assays

VCD, cell viability, glucose and lactate were monitored through daily sampling. VCD and cell viability were measured using a Vi-CELL XR counter (Beckman Coulter). Glucose and lactate were detected using a Cedex Bio HT analyzer (Roche). The off-line testing of osmolality was conducted by using Osmometers (Advanced Instruments). For titer measurements, the cell culture broth was centrifuged at 14,000 *g* for 5 min, and the supernatant was analyzed using an Agilent 1260 HPLC (Agilent Technologies, Santa Clara, CA) with a POROS® A column from Thermo Fisher Scientific.

The supernatant samples were first purified by Protein A chromatography (MabSelect SuRe™ LX resin, Cytiva, Uppsala, Sweden) prior to product quality analysis. Size exclusion chromatography for high and low molecular species was performed on Agilent 1260 HPLC system using a Tosoh TSKgel G3000SW$_{xl}$ column with a 0.5 mL/min flow rate of the mobile phase (20 mM sodium phosphate, 300 mM sodium Chloride, pH 7.4), column temperature at 20 ℃, and UV detection at 280 nm. Both reduced and non-reduced CE-SDS were performed using a Beckman Coulter PA800 Plus instruments equipped with a photodiode array detector set at 220 nm.

Charge variant species were assayed by imaged capillary isoelectric focusing, using a Protein Simple CIEF analyzer with FC-coated iCIEF cartridge. Samples were mixed with appropriate pI markers (5.85/9.50), Pharmalytes (5–8, 8–10.5), 0.35 % methyl cellulose, 1 M urea, 10 mM arginine and iminodiacetic, and then injected into a fluorocarbon coated capillary cartridge. The detection wavelength was set at 280 nm to evaluate the charge variants distributions.

The profile of N-Glycan was characterized by following the procedure of glycan release, purification, 2-AB (2-aminobenzamide) labeling, and UPLC-Fluorescent Light Detection (UPLC-FLD) analysis. The labeled glycans were separated by using a Waters ACQUITY UPLC® BEH Amide column (1.7 μm, 2.1 × 100 mm) and detected by Hydrophilic Interaction Liquid Chromatograph Coupled with Fluorescent Detector (HILIC-FLD) on a Waters Bio-Core system H-class UPLC system with a fluorescent detector. Each individual N-glycan was quantified by its relative peak area using the FLR detector.

2.5. Media cost and COG analysis

For manufacturing COG comparison, all manufacturing costs were calculated under the condition of the 2000 L single-use bioreactor scale. The COG analysis was conducted based on the method described previously [24]. Briefly, the operating costs mainly considered utility and labor related utilization. For the utility part, facility occupancy, equipment usage and its associated maintenance and depreciation, along with the utility charges from water, gas and electricity consumptions, were summarized. In regard to the labor usage, the calculation was mainly determined by the complexity of the operation in each unit operation, with an estimated operation hours and operators needed based on manufacturing experiences. The M&C included both upstream (single-use bioreactors, media, disposable mixing and storage bags, media filters, tubing, probes, and ATF columns) and downstream (buffers, mixing and storage bags, and filtration membranes) materials, the consumption of which were simulated based on the actual usage as per the bill of material lists. Resin costs were not considered in the COG calculation. This is due to the fact that in consecutive commercial manufacturing campaigns, resins can be reused up to a predefined life cycle limit, which leads to an equivalent minimal addition to the overall COG, regardless of the upstream process conditions. As for the media cost comparison, the relative unit price of MagniCHO™ is 60 % of Media A, and the same feed media price was used for all the process conditions.

3. Results

3.1. Productivity improvement via intensified fed-batch process implementation using commercial media

In an initial attempt to improve the productivity of Mabcalin™ production, three commercially available media (Media B–D) were evaluated in shake flasks under both TFB and IFB modes, with Media A as the control from the original TFB process.

In the TFB process as shown in Fig. 3a, Media C and D enabled faster exponential cell growth and reached the highest peak VCD of ~ 30.0 × 10^6 cells/mL on day 5, which was two days earlier than the control. Media B performed the worst in terms of cell growth, which peaked at only 21.3 × 10^6 cells/mL on day 9. Besides, the end VCD in Media B/C/D declined to between 11.0 and 16.6 × 10^6 cells/mL in the late stage of the cultivation, with the viability dropping to 50–73 %. In contrast, the TFB process in Media A showed the highest end VCD and viability of 22.1 × 10^6 cells/mL and 87.5 %, respectively, which also led to the highest titer of 3.9 g/L. Similarly, in the IFB cultures (Fig. 3b), Media A showed the best cell growth potential with the peak VCD climbing to ~ 37.0 × 10^6 cells/mL, comparable to Media C, but apparently better viability maintenance until day 14. In comparison, all the conditions except for the control were harvested earlier (day 10–11) due to the faster viability drop. Consequently, Media A achieved the highest IFB titer of 5.44 g/L (Fig. 3c), the highest IVCD of 389 × 10^6 cells·day/mL (Fig. 3d), and a similar N-glycan profile to the reference standard (Fig. S1). Based on the result, despite a similar TFB titer between Media A and C, Media A outperformed all others under the context of IFB production. As such, Media A was remained as the leading production media for the following IFB study.

Subsequently, the feasibility of switching to an IFB process using Media A was further verified in benchtop 3 L bioreactors. Initially, the potential of cell proliferation under the continuous perfusion mode using an alternating tangential flow (ATF) cell retention device was evaluated. The approach aimed to determine the inoculation density of the IFB production, based on the N-1 cell growth potential. As shown in Fig. 4a, the conventional N-1 batch culture used to seed the TFB production started at an initial VCD of 0.3 × 10^6 cells/mL and reached a final density of 5.2 × 10^6 cells/mL on day 3. By using N-1 perfusion with continuous media exchange from day 2 to day 5, an end VCD of 78.8 × 10^6 cells/mL was obtained. Consequently, the initial seeding density target was able to reach 10.0 × 10^6 cells/mL (Fig. 4b). During production, daily bolus feeding was performed, with the total feed volume reaching 36.3 % of the initial working volume in the IFB process, compared to 33.5 % in the TFB control. Following temperature shift on day 2, the VCD of the IFB culture continued to rise to 42.4 × 10^6 cells/mL on day 5, in contrast to 31.2 × 10^6 cells/mL on day 8 in the TFB production. In terms of viability, both of the IFB and TFB conditions shared a similar trend until day 6. After day 6, the viability of the IFB culture showed a faster decline to 63.0 % in comparison to 91.5 % in the TFB on day 14 (Fig. 4b). Concurrent with the drop in viability, lactate in the IFB culture also trended higher towards the end of the culture. The end lactate of the IFB production rose to 1.44 g/L on day 14 compared to 0.62 g/L in the TFB (Fig. 4c). The 58.2 % higher IVCD compensated the slightly lower cell specific productivity (Qp) observed in the IFB production (Fig. 4d). Eventually, the titer of the IFB culture increased to 6.1 g/L, which was 156.4 % of the TFB control. The IFB results indicated that fed-batch intensification through N-1 perfusion was a feasible approach to improve productivity, even though some undesirable performance, such as faster cell viability decline and lactate re-accumulation, started to emerge in the same time.

3.2. Productivity improvement via the application of proprietary MagniCHO™ media

The performance of an IFB culture highly relies on the nutritional

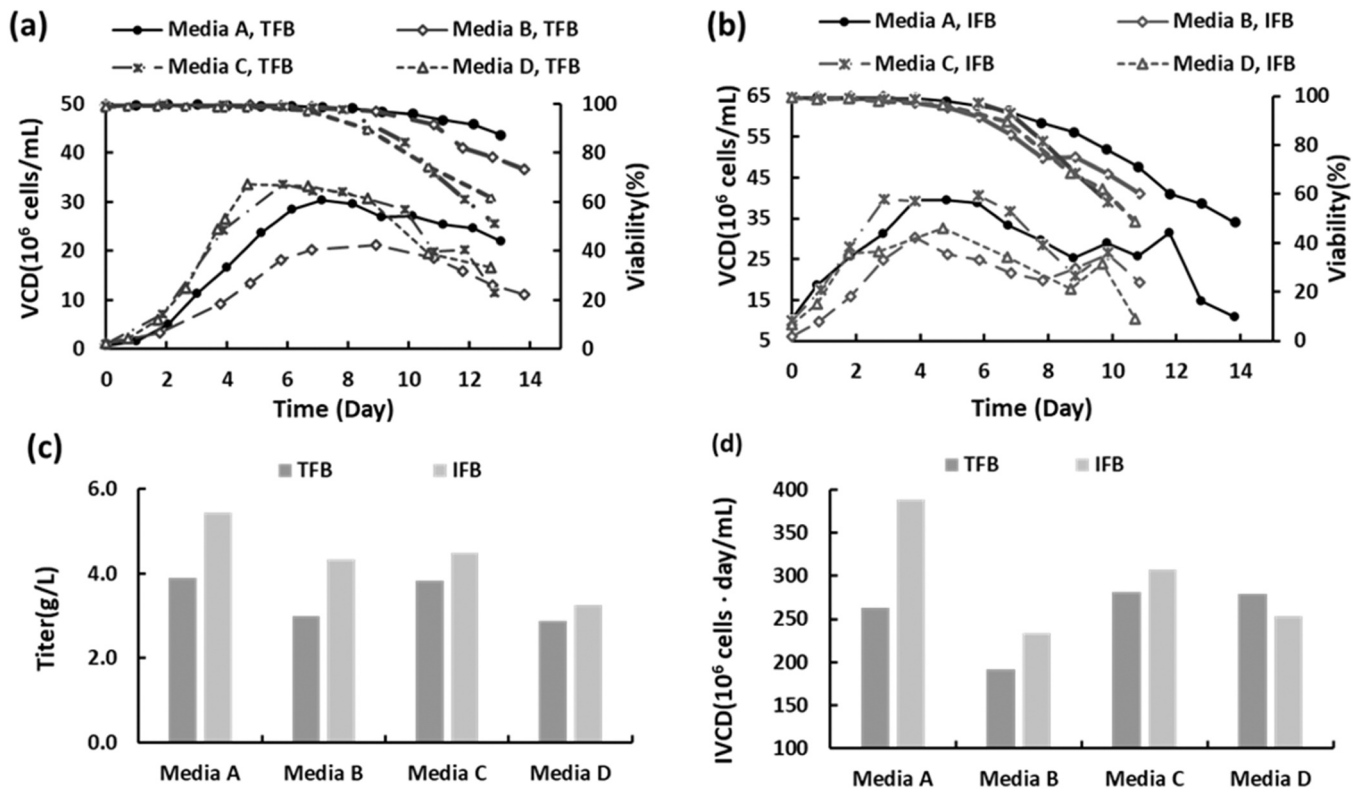

Fig. 3. Culture performance with candidate commercial media in shake flasks; VCD and cell viability in (a) TFB and (b) IFB; (c) Titer; (d) IVCD.

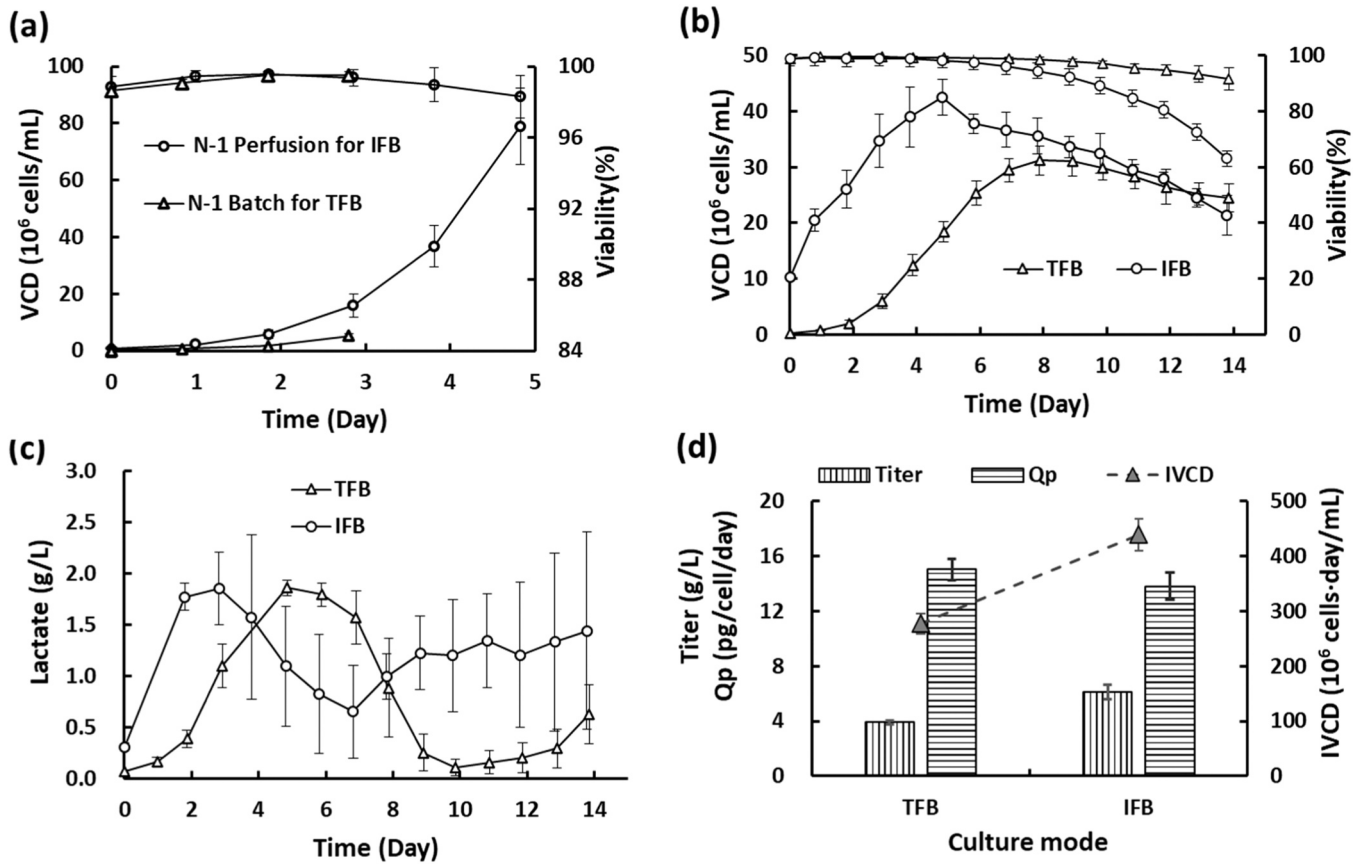

Fig. 4. Culture performance in 3 L bioreactor with selected media A, (a) N-1 VCD and cell viability; (b) Production VCD and cell viability; (c) Lactate; (d) IVCD, titer, and Qp.

capacity and balance of the cell culture media for high cell density maintenance. In the meantime, an appropriate medium for intensified processes is capable of restraining or counteracting unfavorable effects from by-product synthesis. To further improve the medium performance in the intensified fed-batch production of Mabcalin™, Media A was enriched with either the feed media or Pluronic F68 (a surfactant protects cells from shear stress). However, neither showed significant performance improvement (Fig. S2). Based on the results, a proprietary production medium, MagniCHO™, specifically designed for intensified cell culture processes, was evaluated in this round of IFB optimization. A side-by-side evaluation of MagniCHO™ and Media A was performed to compare their performance in both N-1 perfusion and IFB production.

In the N-1 perfusion culture, the cell growth profile using the two different media started to differentiate from day 4 onwards: the peak VCD climbed to 86.8 × 10^6 cells/mL on day 5 with MagniCHO™, compared to 78.8 × 10^6 cells/mL with Media A (Fig. 5a). Simultaneously, the viability with Media A slowly trended downwards to 98.3 %, compared to 99.2 % in MagniCHO™, which implied an emerging sign of cell apoptosis under the Media A condition. Moreover, even with a higher end VCD with MagniCHO™, the total N-1 perfusion volume summed up to only 4.5 times of the vessel volume, which was much lower than the Media A control (6.0 times of vessel volume). The result revealed that using MagniCHO™ as the perfusion medium was able to provide improved proliferation performance and better sustained viability, while reducing total media consumption and costs.

Following N-1 perfusion, the IFB-1 production using Media A and the IFB-2 using MagniCHO™ were inoculated separately by keeping the same initial cell density target of 10.0 × 10^6 cells/mL. After inoculation, a similar daily bolus feeding schedule was applied for the comparison of the two different processes. During the early stage of the production, MagniCHO™ contributed to a higher peak VCD of 51.7 × 10^6 cells/mL vs. 42.4 × 10^6 cells/mL with Media A (Fig. 5b). A marked difference in viability appeared towards the end of the production: the viability started to drop rapidly in IFB-1 to as low as 63.0 % on day 14, while higher than 80 % viability was observed on day 14 in the IFB-2 condition. Meanwhile, the lactate profile of IFB-1 also exhibited a second shift towards re-accumulation from day 7, while only a negligible amount of lactate remained in the end when MagniCHO™ was used (Fig. 5c). As a result of the enhanced metabolic status with the use of MagniCHO™, the final titer increased from 6.1 g/L in IFB-1 to 9.1 g/L in IFB-2 (Fig. 5d), which was driven by the improvement in both IVCD and cell specific productivity (Qp) by 23.9 % and 21.4 %, respectively.

3.3. *Productivity improvement via the ultra-intensified IPFB process (UI-IPFB)*

To further explore alternative intensification strategies to enhance productivity, the UI-IPFB cell culture process was applied using either Media A or MagniCHO™ as both the production and the intermittent perfusion media. In the UI-IPFB study with MagniCHO™, the seeding densities ranged from 20 to 80 × 10^6 cells/mL under different perfusion rates were preliminarily evaluated in Fig. S3. Among these conditions, the highest seeding density of 73.6 × 10^6 cells/mL from five-fold N-1 seed concentration was selected as the best condition for the following comparison. It was found that the end VCD of UI-IPFB process reached as high as 372.9 × 10^6 cell/mL after five-fold concentration of the N-1 perfusion culture (Fig. 6a). During production, three separate cycles of 24-hour intermittent perfusion on day 4, 7 and 10 were performed. In contrast, only marginal protein production was detected in the IPFB production using Media A, due to aberrant lactate accumulation throughout the production phase (data not shown). Therefore, MagniCHO™ was chosen for the UI-IPFB process.

During the UI-IPFB production, the peak VCD reached 101.5 × 10^6 cells/mL on day 2 (Fig. 6b). Despite the much higher seeding density, the UI-IPFB culture exhibited a comparable viability profile to the IFB-2 process, with the viability slowly declining to 84.0 % on day 14 and further to 79.4 % after extension to day 16 (Fig. 6c). Given that the titer continued to trend up after day 14 (trend not shown), the production

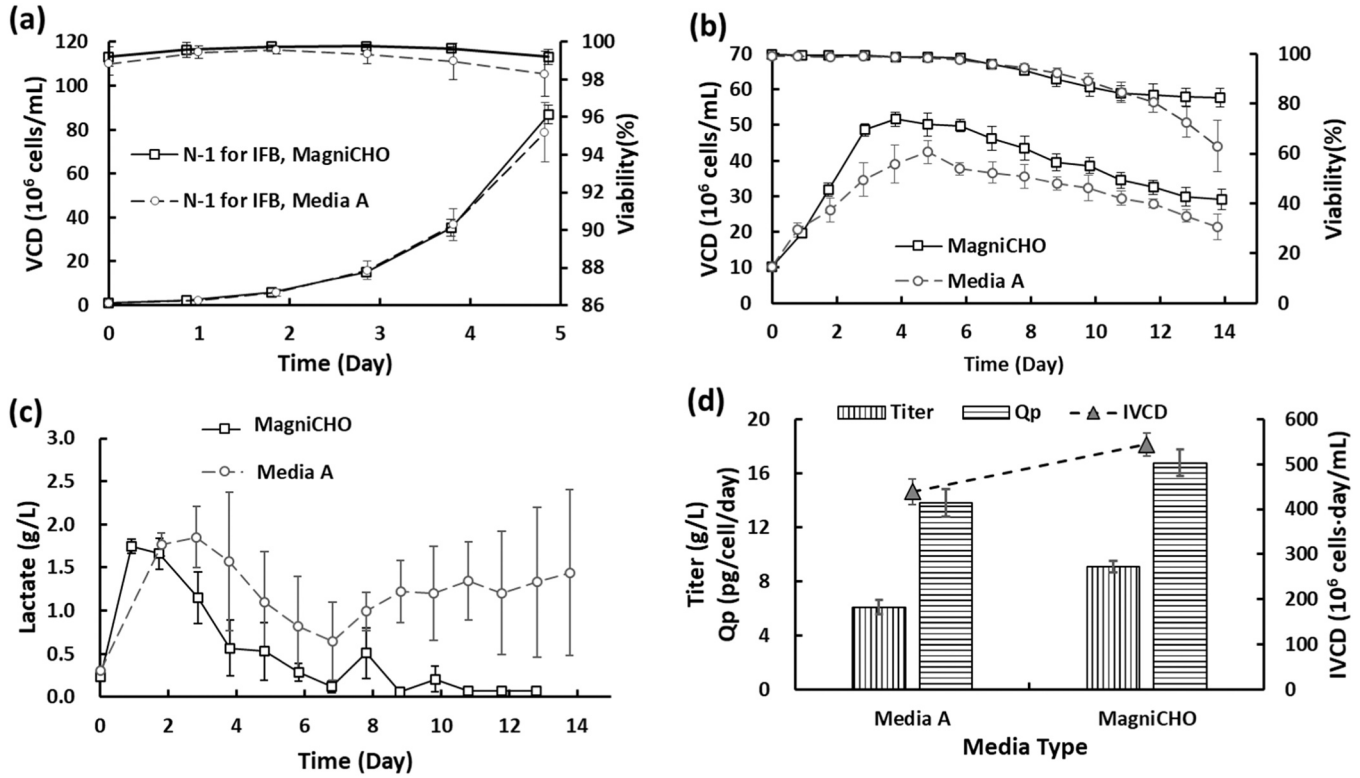

Fig. 5. IFB production using Media A (IFB-1) and MagniCHO™ (IFB-2). (a) N-1 VCD and cell viability; (b) Production VCD and cell viability; (c) Lactate; (d) IVCD, titer and Qp.

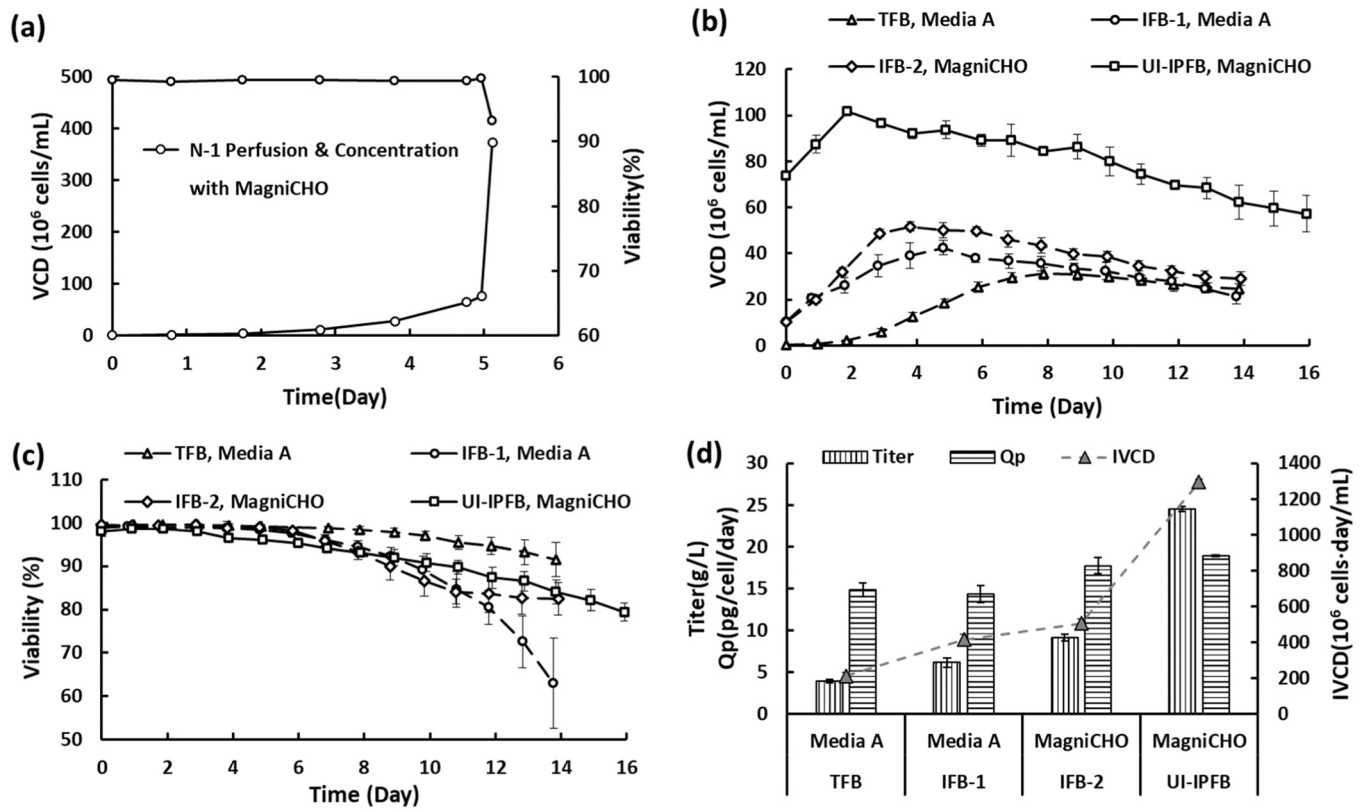

Fig. 6. Comparison of TFB, IFB and UI-IPFB performance using Media A or MagniCHO™ media. (a) N-1 VCD and cell viability; (b) Production VCD; (c) cell viability; (d) IVCD, titer and Qp.

duration was extended to day 16. At the end of the production, the final product titer of 24.5 g/L was obtained, which corresponded to 6.3 folds of the TFB titer with Media A, and 2.7 folds of IFB-2 with MagniCHO™. The dramatic increase in titer was partly because of the notable surge in IVCD by 508 % and 157 % relative to the TFB and IFB-2 conditions, respectively (Fig. 6d). Additionally, the Qp calculated from the UI-IPFB culture showed a modest improvement to 18.9 pg/cell/day from 14.8 pg/cell/day in the TFB control. However, the difference was marginal between the IFB-2 and UI-IPFB conditions (Fig. 6d).

In terms of product quality, Fig. 7 summarized the results from different processes with the TFB condition as the control. The percentages of high and low molecular weight species (HMW and LMW), as determined by SEC-HPLC, were highly comparable, showing a similar ratio of aggregates (< 5 %) and negligible amounts of truncated species. The similarity in regard to product aggregation and truncation was further revealed from the CE results. Regarding charge distributions, some minor differences were observed, as the acidic species from all three intensified processes showed a slight increase ranging from 0.6 % to 4.0 %. Besides, the N-glycan analysis indicated a decrease in both galactosylation and mannose levels with process intensification, resulting in 7.5–10.2 % higher G0F population in both IFB and UI-IPFB products.

3.4. Media cost and COG analysis

To provide a comprehensive overview of manufacturing cost distributions and COG reduction projections, a systematic cost analysis was performed based on the different processes developed. Initially, we calculated the media costs for the different culture modes using either commercial Media A or MagniCHO™ to demonstrate how the evolution of process modes and the selection of an optimal media could benefit the final drug substance (DS) output per batch and the media expense per unit DS product (Table 1 and Fig. 8).

Assuming in a 2000 L single-use bioreactor (SUB), the DS output from the original TFB process using Media A could reach 5.1 kg. By opting for a better media option, such as MagniCHO™, the DS production increased to 6.5 kg per batch, due to the 28 % higher TFB titer with MagniCHO™ adoption. Furthermore, the improvement in production

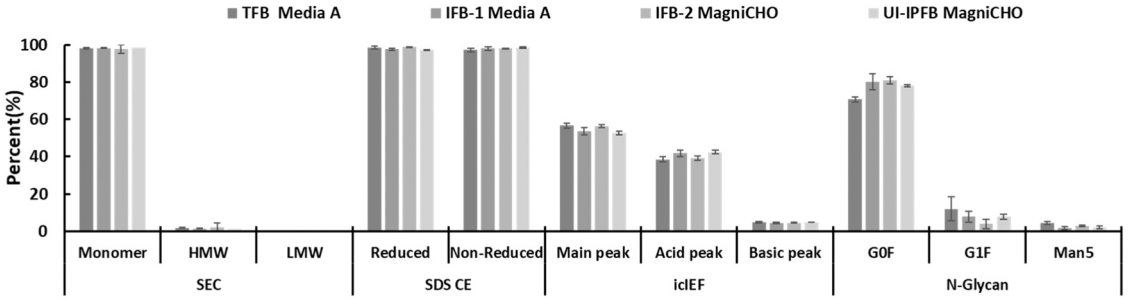

Fig. 7. Product quality comparison with different processes.

Table 1
Media cost comparison in different processes.

Process mode Parameters	TFB Media A	TFB MagniCHO™	IFB-1 Media A	IFB-2 MagniCHO™	UI-IPFB MagniCHO™
BR scale (L)	2000	2000	2000	2000	2000
N duration (day)	14	14	14	14	16
Titer (g/L)	3.9	5.0	6.1	9.1	24.5
DSP yield (%)	65	65	65	65	65
DS Amount (g)	5070	6500	7930	11,830	31,850
BM (L)	1700	1700	3200	2500	9450
FM1 (L)	504	504	474	420	720
FM2 (L)	50.4	50.4	47.4	42	72
Normalized Media fee %	100 %	53 %	91 %	30 %	28 %

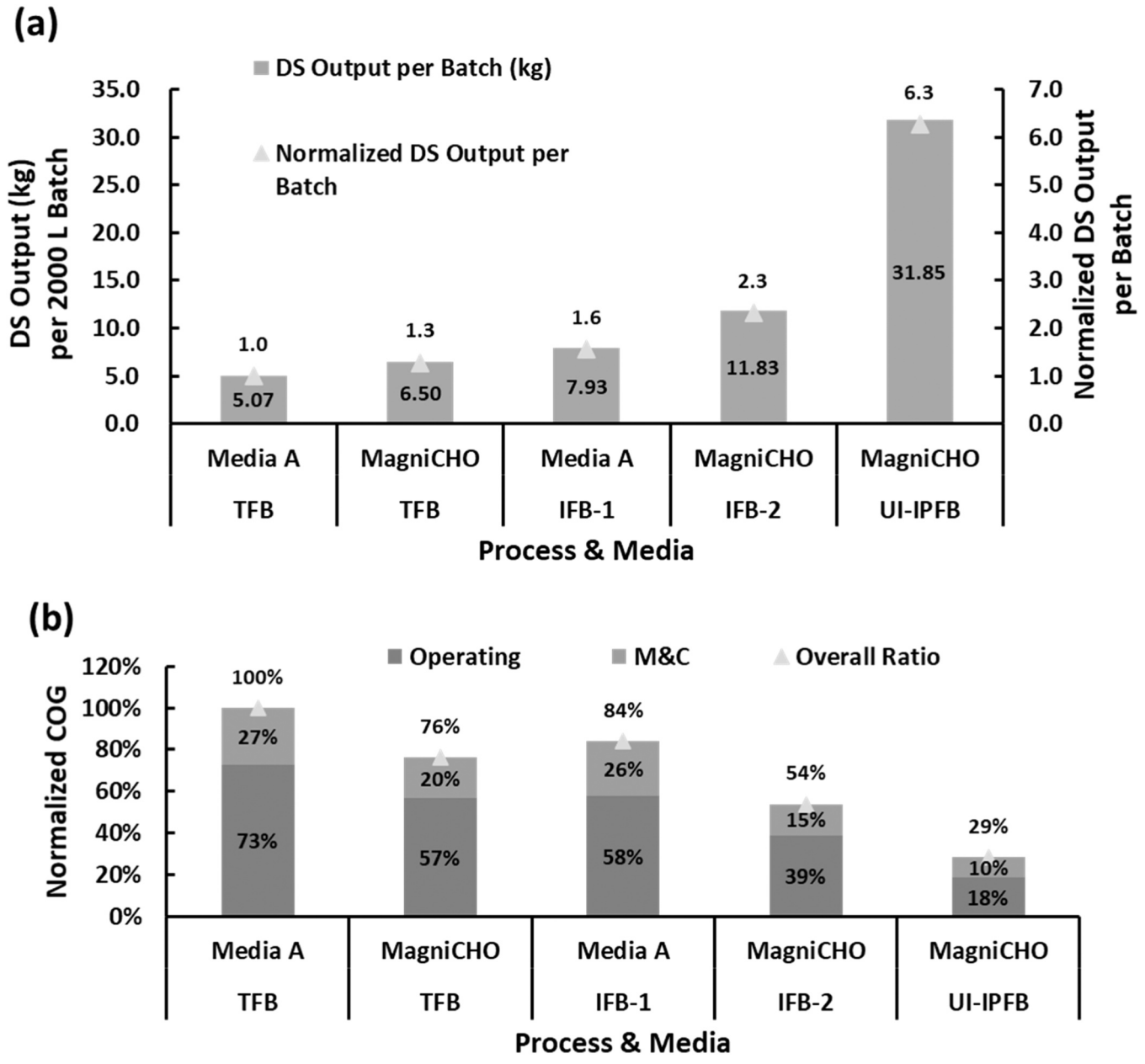

Fig. 8. Process output and economy analysis with different conditions. (a) DS outputs per batch and normalized ratios; (b). Normalized COG with breakdown ratios.

efficiency became more pronounced with the optimized IFB processes, in which the DS production climbed up to 11.8 kg per batch in IFB-2, which was 133 % higher than the TFB control process with Media A. Greatly surpassing the IFB output, UI-IPFB with MagniCHO™ produced 31.9 kg of the DS, which was 6.3 folds of the TFB control (Table 1 and Fig. 8).

The assessment in Table 1 also revealed that using the proprietary media MagniCHO™ could lead to significant savings in media expenses.

Firstly, the conversion from Media A to MagniCHO™ in the TFB process not only increased the titer by 28 %, but also lowered the final media cost per DS to 53 % of the control, thanks to the additional 60 % reduction in the MagniCHO™ unit price. After applying the IFB process, a low-performing media choice of Media A provided only marginal benefits in media cost savings with the specific media cost reduced to 91 % of TFB. In other words, although the titer of the IFB-1 production increased to 6.1 g/L, the considerably higher media consumption as a result of N-1 perfusion did not lead to more competitive media savings. In contrast, the specific media costs were slashed by 70 % in both IFB-2 and UI-IPFB processes, owing to the improved productivity achieved by using the MagniCHO™ media.

Under the same TFB production mode, the COG reduction largely hinged on the comparative media performance between Media A and MagniCHO™. Assuming that the downstream processing yield was similar across all the conditions studied, the normalized COG could decrease to 76 % of the control by simply replacing the media from commercial Media A to MagniCHO™ in the TFB production (Fig. 8b). In addition, process intensification using the IFB processes was also proven to be effective, notably driving down manufacturing COG by 46 % in the IFB-2 process, but to a less degree of merely 16 % in the IFB-1 process. This further underscored the essential role of cell culture media in maximizing the potential of intensified process strategy on productivity improvement and COG reduction. More significantly, an ultra-intensified UI-IPFB process was developed which realized 5.3-fold increase in titer and 71 % reduction in COG (Fig. 8b). As demonstrated, through our stepwise process intensification and optimization strategy, the evolved process performance from TFB to the more advanced IFB and UI-IPFB processes also translated into significant savings in the manufacturing COG.

To provide a systematic evaluation on manufacturing costs, the COG calculation was divided into two main components to reflect the individual contributions from operating costs and material & consumable (M&C) expenses (Fig. 8b). In the TFB control process, the operating cost constituted a large portion (73 %) of the total costs, with the M&C expenses accounting for the remaining 27 %. The distribution highlighted a significant contribution from the operating-related utility and labor utilization in the baseline TFB process. However, with the application of MagniCHO™ in the TFB process, a noticeable shift in the COG contribution was observed, with most of the COG reduction attributed to the operation related saving from 73 % to 57 %, mainly due to the higher DS generation without much changes in operation complexity. Transitioning to the IFB-2 production using the MagniCHO™ media exhibited a more prominent COG reduction in both of the operating and M&C elements, which ended up with 39 % and 15 % of the TFB control, respectively. Ultimately, the UI-IPFB process demonstrated the most substantial COG reduction among all the conditions studied, with the operating costs slashed to 18 %, and the M&C to only 10 % of the control. The detailed cost distribution analysis illustrated how the stepwise process intensification and optimization could enable increasing COG reduction, primarily through enhanced manufacturing operation efficiency.

4. Discussion

The manufacturing COG plays a crucial role in determining patient affordability, thereby influencing the economic viability of the drug in comparison to its market competitors. Our COG analysis pointed out operating costs and M&C expenses as the major cost contributors in biopharmaceutical manufacturing.

Generally, the operating cost consists of utility- and labor-related elements. It was shown in this study that utility costs carried the most significant weight among all the cost categories, which was also demonstrated in prior studies [36,37]. The prominence of utility costs is particularly noticeable, partly due to the high-standard Good Manufacturing Practices (GMP) requirements for specialized and validated equipment, high-grade materials, and advanced facilities [11]. Meanwhile, the utility expenses also involve considerable amounts of electricity, water and gas, especially in facilities that operate 24/7 to meet production demands [38]. The intensification of cell culture processes was proven to be pivotal to the reduction of the utility costs per DS production, although more sophisticated equipment, such as ATF, and possible heavier product purification loads may be inevitable. However, advances in process intensification have reported to result in higher upstream yields, shorter production times or manufacturing cadence [39]. These benefits compensate for the potentially higher overall operating costs usually involved in an intensified process, leading to enhanced operation efficiency and reduced COG readout [40].

In this study, we demonstrated the effectiveness of process intensification on driving down the relative operating costs from 73 % of TFB to as low as 18 % in the UI-IPFB condition, although the implementation of UI-IPFB process may involve increased labor usage, equipment and facility utilization, such as ATF perfusion devices in both N-1 and production stages, additional media and buffer preparation, and larger space occupancy for storage tanks or bags for higher volumes of intermediates and DS. In addition, the transition from the TFB process to the more advanced cell culture processes like IFB and UI-IPFB usually incurs a direct uptick in total M&C costs as well. The increase could be attributed to the adoption of perfusion technology which leads to increased usage of media and ATF filters during upstream processing. Moreover, the enhancement of upstream productivities necessitates more extensive downstream operations, further escalating costs. Therefore, it may be a potential challenge to balance the quest for higher productivity with the imperative to manage and mitigate M&C expenses [41]. One common countermeasure lies in the evaluation and adoption of more cost-effective materials, including alternative media or consumables, as exemplified by the replacement of Media A with MagniCHO™ in this case. Thus, by leveraging different cost-benefit materials-not only cell culture media but also low-cost filters, bags, resins, or chemicals-the challenge of high M&C costs could be addressed directly. Alternatively, many technological innovations, such as intensified harvest with acidification or flocculation [42,43], advanced chromatography polishing with weak partitioning or overloading modes [44,45], integrated inline conditioning or dilution system [46], have been reported to enable improved downstream processing capacity and reduced M&C usage [47,48]. All these advancements further align with the broader objective of enhancing the economic and environmental sustainability of biomanufacturing operations.

It should be noted that, based on our practical knowledge, the overall upstream media costs incurred with the use of different production process modes could add up to 20–60 % of the total M&C expenses. Therefore, media screening and optimization are often an integral part of process development efforts. To highlight the significance of cell culture media on overall manufacturing economy, a head-to-head media cost comparison was conducted among different process scenarios. MagniCHO™ replacement could lead to 47 % media cost reduction under the same TFB process mode, demonstrating the advantages of applying optimized proprietary media in a cell culture process over commercial options. The effect of the media optimization strategy became even more outstanding when combined with intensified processes. As shown in the IFB and UI-IPFB condition, the use of MagniCHO™ lowered the relative media cost per DS to 28–30 % of the original TFB process.

In addition, our analysis uncovered an indirect relationship between total media consumption and DS specific media costs. Although continuous cultivation, such as Concentrated Fed-Batch (CFB) and Intensified Perfusion Culture (IPC), are widely known to support increased cell biomass accumulation and enhanced daily productivity [49], our study found that the continuous approach was not a viable solution for the production of this molecular, as evidenced by the low productivity and poorly regulated lactate metabolism in the CFB production (Fig. S4). Indeed, in certain cases, continuous manufacturing

could not guarantee economic competitiveness, due to the large usage of media, low clarification yield, and extended facility operation [50]. UI-IPFB production provides an alternative intensification strategy, which circumvents the common drawbacks in a continuous production, and meanwhile takes the advantages of perfusion cultivation for better cell status maintenance. In this study, even though the UI-IPFB process required the highest amount of media, its medium cost efficiency outperformed all other conditions, given the much higher titer it achieved. This indicated that process intensification, despite triggering a higher expense budget for materials and consumables, was proven to be a highly value-added practice in the development of a cost-effective manufacturing process.

Despite the advantage of UI-IPFB in achieving high cell density and titer, the challenge of developing a scalable UI-IPFB process should not be underestimated. On the one hand, the N-1 step of UI-IPFB gives rise to a highly concentrated cell density, which may trigger excessive pCO_2 and foam accumulation during the N-1 stage. Therefore, an enhanced CO_2 stripping strategy could be designed with proper foam control. Besides, choosing an appropriate number of ATFs with a suitable size (e.g., a larger XCell ATF 10 system) based on a similar filter flux and ATF-to-filtration ratio is necessary to achieve the same concentration factor in a similar time frame to the small-scale process. Based on our experience, due to high viability and minimal cell debris at N-1 perfusion stage, the ATF hollow fiber could resist a relatively high flux ratio without the fouling risk. Additionally, the concentration factor depends on the minimal working volume that a bioreactor vessel could support. For that, as high as 20:1 turndown ratio of commercially available single-use bioreactors has been engineered to enable a low working volume in a large vessel. On the other hand, sustaining a high cell density culture during an UI-IPFB production is inevitable, leading to a high oxygen transfer rate (OTR) requirement. However, it has been reported that a volumetric oxygen mass transfer coefficient (kLa) up to 40 h^{-1} could be reached at a 2000 L SUB [51], making the ultra-high cell density production attainable. Also, one who choose to apply UI-IPFB in production may consider modifying bioreactor equipment and facility layout to incorporate the perfusion function in both the N-1 and production stages, although the requirement tends to be much simpler than those needed for fully continuous perfusion, such as CFB or IPC. Furthermore, the indication to downstream purification needs to be extensively evaluated [52], as potential adverse impacts on product qualities may require special attentions, such as residual HCP causing polysorbate degradation and particle formation in final formulations [53,54], and product-related impurities formed from degradation or aggregation [55,56]. Therefore, optimizing the downstream process to better fit for the intensified production is recommended. In spite of the challenges, the scale-up of UI-IPFB has been proven in both 250 L and 2000 L SUB at WuXi Biologics, producing comparable product quality with an appropriate downstream purification strategy.

It is also worth noting that inappropriately applying a less optimal medium in an intensified process may become counterproductive in an attempt to achieve better process productivity and COG reduction, as observed in the IFB-1 and the UI-IPFB processes using Media A in this study. In the IFB-1 with the Media A condition, the titer was raised to 6.1 g/L, leading to a COG reduction by merely 16 %, which could not even be compared to the 24 % COG reduction in the TFB process using MagniCHO™. Similarly, a previous study found that the applying IFB production while maintaining the same original fed-batch media resulted in only comparable titers and a limited COG reduction of 7–9 % [57]. Furthermore, the use of Media A in the UI-IPFB process directly led to severely suppressed lactate consumption throughout the production period, and therefore only marginal product was detected in the end (data not shown). The lactate spike observed with Media A reiterated the importance of media optimization to mitigate such metabolic byproduct accumulation, which can adversely affect cell viability and titer. The findings suggested that although applying an intensified process, such as UI-IPFB, has been broadly utilized for titer improvement, the optimization of the cell culture medium was equally important for achieving well-sustained cell growth and longevity, balanced and controlled cell metabolism, and consequently, improved product synthesis to maximize the effect of process intensification on manufacturing efficiency.

Many studies reported that tailored media formulations that meet the specific nutritional requirements of a cell line could lead to higher yields [58,59]. Specifically, MagniCHO™ development journey began with a series of DoE screening studies. This iterative formulation optimization allowed for the identification of key factors that significantly impacted the media performance especially under an intensified process. Concurrently, spent medium analysis could provide further insights on how to approach a relative nutritional balance at the cellular level under different metabolic stages. Based on the analysis, the concentrations of amino acids were carefully determined. Meanwhile, various factors including vitamins, reducing agents, metals and chelators, growth enhancers and nucleotides, which are known to counteract reactive oxygen species (ROS) damage and support essential cell metabolism, were meticulously studied and tuned to minimize late-stage unfavorable metabolic outcomes. The resulted formulation was then validated through rigorous testing across different culture modes to ensure it can meet the desired performance for cell growth and productivity. In this study, the replacement from Media A to MagniCHO™ in the TFB process resulted in an increase in titer from 3.9 g/L to 5.0 g/L. This improvement, representing a 28 % increase in titer and 26 % decrease in COG. Likewise, in the N-1 perfusion stage of the IFB process, MagniCHO™ was able to support a higher end VCD with 25 % less media usage, compared to Media A. Besides, the IFB titer increased from 6.1 g/L to 9.1 g/L, following the implementation of MagniCHO™ media. Even with an ultra-high seeding of 80 $\times$ 10^6 cells/mL in the UI-IPFB production, the competitive advantage of MagniCHO™ become more significant, achieving the highest titer of 24.5 g/L in this study, in contrast to negligible product detection in the Media A condition. The substantial improvement not only raised the titer benchmark of CHO-based bioproduction through either a fed-batch or a hybrid process, but also substantiated the significant role of process intensification and media optimization in catalyzing a paradigm shift towards more cost-effective biologics manufacturing. Moreover, the stepwise process intensification to improve titers and lower COG is also in accordance with the drug development trajectory from clinical to commercial manufacturing. In the early stage, a more common TFB process may be sufficient for clinical safety and efficacy demonstration, and an intensified process may outrun the early-stage clinical demand with additional resource and facility investment. But as the clinical trial proceeds, process intensification may take center stage, as the process output and economy become central to meeting commercial market forecast. In the meantime, knowing the facility capability under different processes and choosing the best commercial process maximizing manufacturing efficiency would necessitate a comprehensive assessment on a case-by-case scenario.

5. Conclusion

The adoption of cell culture intensification strategies has been recognized as an effective approach to elevate productivities while simultaneously lowering manufacturing COG. The study showcased a classic and evolving practice of process intensification and media optimization, through the implementation of IFB and UI-IPFB process strategies alongside an optimized proprietary cell culture medium. The strategy notably improved the cell culture titer to 24.5 g/L in a 16-day fed-batch production, marking a significant increase of more than 6 folds over the original TFB process. Moreover, employing the UI-IPFB process promoted a substantial COG reduction of 71 % in a 2000 L manufacturing scale. The application of the UI-IPFB strategy coupling with an optimized media formulation has the potential to advance the conventional fed-batch production of biologics, making it more cost-

effective, sustainable, and productive. This shift not only benefits the biomanufacturing industry by improving efficiency and reducing COG, but also has a profound impact on global healthcare by making life-saving biologics more accessible to patients worldwide.

CRediT authorship contribution statement

Sebastian Hogl: Writing – review & editing, Validation. **Jun Tian:** Writing – review & editing, Supervision. **Kecui Xu:** Investigation, Formal analysis, Data curation. **Yanyan Cui:** Investigation, Formal analysis, Data curation. **Le Yu:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Weijia Cao:** Writing – review & editing, Writing – original draft, Project administration, Formal analysis, Conceptualization. **Jinliang Zhang:** Writing – original draft, Methodology, Formal analysis, Conceptualization. **Sherry Gu:** Writing – review & editing, Supervision. **Weichang Zhou:** Writing – review & editing, Supervision. **Hitto Kaufmann:** Writing – review & editing, Validation.

Abbreviations

ATF	Alternating Tangential Flow Filtration
BsAb	Bispecific Antibody
CFB	Concentrated Fed Batch
CHO	Chinese Hamster Ovary
COG	Cost of Goods
DS	Drug Substance
HMW	High Molecular Weight Species
IFB	Intensified Fed-Batch
IVCD	The cumulative Integral of Viable Cell Density
LMW	Low Molecular Weight Species
mAb	Monoclonal Antibody
M&C	Materials and Consumables
OTR	Oxygen Transfer Rate
Qp	Cell Specific Productivity
SUB	Single Use Bioreactor
TFB	Traditional Fed-Batch
UI-IPFB	Ultra-Intensified Intermittent-Perfusion Fed-Batch
VCD	Viable Cell Density
VVD	Vessel Volume each Day

Author Contributions

Jinliang Zhang and Le Yu: conceptualization and study design; Yanyan Cui and Kecui Xu: study execution; Jinliang Zhang and Weijia Cao: data analysis and interpretation; Jinliang Zhang, Weijia Cao and Le Yu: manuscript writing; Jun Tian, Sebastian Hogl, Hitto Kaufmann, Weichang Zhou and Sherry Gu: manuscript review.

Disclosure of Interest

The authors report no conflict of interest.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2024.109476.

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