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Advances in Perfusion Culture Processes for Mammalian Cells: Postprint

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Abstract

Currently, the biopharmaceutical sector faces numerous challenges to existing biomanufacturing technologies due to cost pressures, sharp fluctuations in market demand, and increasingly intense competition from biosimilars. Consequently, biotechnology companies are increasingly inclined to develop flexible, efficient, and innovative manufacturing processes. Perfusion culture, as one of the key processes in contemporary mammalian cell culture, not only provides a stable environment favorable to cells by continuously removing byproducts and replenishing nutrients, thereby addressing issues such as unstable protein quality or low expression levels, but also enhances capacity utilization and production efficiency by increasing volumetric productivity. This article systematically reviews the research progress on perfusion culture for mammalian cell culture and provides a reference for its further development and application.

Full Text

Research Progress in Perfusion Culture Processes for Mammalian Cells

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Abstract

In the current biopharmaceutical landscape, existing bio-manufacturing technologies face significant challenges due to cost pressures, rapidly fluctuating market demands, and intensifying competition from biosimilars. Consequently, biotechnology companies are increasingly motivated to develop innovative, flexible, and cost-effective manufacturing processes. Perfusion culture, as a key technique in mammalian cell culture, offers distinct advantages: it provides a

stable, cell-friendly environment by continuously removing by-products and replenishing nutrients, thereby addressing issues such as protein quality instability and low expression levels, while also optimizing capacity utilization and enhancing production efficiency through increased volumetric productivity. This paper systematically reviews the research progress of perfusion culture in mammalian cell culture and provides insights for its further development and application.

Keywords: perfusion culture; mammalian cells; culture process; continuous process

1. Background

Over 900 biopharmaceutical products are currently in clinical development in the United States and Europe, with the majority produced in mammalian cell culture systems. Moreover, the production of humanized recombinant proteins can only be achieved through mammalian cell culture, making the development of mammalian cell culture processes particularly critical for the biopharmaceutical industry [1][2].

Currently, the primary culture modes in bio-manufacturing are batch and fed-batch processes. However, most biopharmaceutical molecules are unstable, and during batch and fed-batch cultivation, cellular metabolic by-products, enzymes released from dead cells, and increased osmotic pressure adversely affect both cell growth and protein quality [3]. As research into biopharmaceutical processes deepens, continuous integrated production of therapeutic proteins is gradually replacing traditional batch cultivation in laboratory, clinical, and commercial manufacturing settings [4]. Regulatory agencies such as the FDA encourage process improvements and innovations, including continuous manufacturing—a shift favored by both emerging technologies and biosimilar developers [5].

Perfusion culture provides a stable and favorable environment for cells by continuously removing by-products while replenishing nutrients. Compared to batch and fed-batch processes, perfusion culture can maintain a stable environment for extended periods at high cell densities while reducing product residence time in the medium, which benefits product quality [6]. Ahn et al. [7] found that perfusion culture minimizes the impact of dynamic changes in medium composition on protein glycosylation, demonstrating its advantage in enhancing product quality.

Perfusion culture is not a novel technology for cell cultivation. Since the 1990s, many commercial biopharmaceuticals have employed perfusion processes, including abciximab, β-glucocerebrosidase, infliximab, basiliximab, interferon β-1α, recombinant human coagulation factor VIII, and golimumab [8].

Traditional perfusion culture often relied on microcarriers and microencapsulation methods for continuous perfusion [9][10][11]. In China, successful industrial applications of traditional perfusion processes have been achieved; for example, the drug Pro-urokinase (recombinant human prourokinase for injection) was

produced at large scale using serum-free continuous perfusion culture for the treatment of acute ST-segment elevation myocardial infarction, and was successfully launched in 2011 [12].

In today’ s diverse manufacturing landscape, biotechnology companies are increasingly inclined to develop highly flexible and efficient production processes [9]. Perfusion culture has become widely adopted in bioengineering as an effective means to enhance the yield of recombinant proteins with low stability [6]. By enabling production levels comparable to large-scale fed-batch processes using smaller bioreactors, perfusion culture achieves process miniaturization and increases operational flexibility [14].

2.1 Cell Retention Systems

The robustness of separation devices is critical for effective cell separation and achieving high cell densities [6][14][15]. Currently, two primary cell retention systems are used for perfusion culture: tangential flow filtration (TFF) and alternating tangential flow filtration (ATF). In TFF, cell suspension is driven by a peristaltic pump to create continuous circular flow; as it enters the hollow fiber membrane, waste medium permeates through the membrane while cells are retained and recirculated back into the bioreactor. ATF employs a diaphragm pump to generate reciprocating suction and pressure, enabling medium to flow alternately through the retention device while metabolic waste passes through the membrane and cells remain in the reactor. The alternating motion in ATF creates a scouring effect on the filtration membrane, helping to prevent fiber clogging [15]. Additionally, ATF offers advantages in improving volumetric productivity; Bosco B et al. [16] demonstrated that replacing internal spin filters (ISF) with ATF increased volumetric productivity by 50-70%.

ATF is currently the more widely adopted method; however, some studies have found it less advantageous than TFF for achieving high cell densities. According to Clincke et al. [17], both ATF and TFF can sustain cultures for two weeks at densities of 20-30$\times10^{6}$ cells/mL, but at high densities of 90-100$\times10^{6}$ cells/mL, ATF can maintain culture for only four days whereas TFF can sustain it for two weeks. Therefore, the selection between ATF and TFF requires comprehensive consideration of cell compatibility.

Novel cell retention devices for perfusion culture are also emerging. Kwon et al. [18] introduced a microfluidic cell retention system based on inertial separation for suspension mammalian cell perfusion culture, though this device is currently limited to laboratory-scale applications.

2.2 N-Stage Perfusion Cell Culture

In N-stage bioreactors (production-scale bioreactors), cell mass can rapidly reach system capacity limits due to nutrient or equipment constraints, leading to a swift decline in cell viability [4]. When using perfusion culture in N-stage

bioreactors, effective control of cell density can prevent this issue and maintain stable cell mass over extended production periods. Cell density is typically controlled through semi-continuous or continuous cell bleeding. Karst et al. [19] used TFF and ATF to maintain cell densities at three stable stages—20, 40, and 60×10^{6} cells/mL—each lasting over one week through continuous cell bleeding.

2.3 Integrated Continuous Operation

As bioreactor perfusion culture integrates with downstream purification, continuous operations have been extensively studied, with their advantages becoming increasingly evident, particularly for capture and purification processes. Given the high cost of resins, maximizing resin utilization in capture steps is crucial. Steinebach et al. [20] introduced a dual-column capture system for continuous processing of cell culture harvest, achieving a 2.5-fold improvement in resin utilization for affinity capture compared to batch-based continuous capture. This system also enables prediction of process performance metrics such as yield, productivity, and capacity utilization. Angelo et al. [21] recently confirmed these performance improvements at pilot scale. Steinebach et al. [23] further demonstrated that perfusion culture with continuous capture processes enables stable production of higher-purity proteins.

2.4.1 Cell Banking and Seed Culture

Reducing seed culture time is a critical aspect of process optimization [22]. To shorten the time required to reach target viable cell density when inoculating N-stage bioreactors, increasing the initial cell mass is an effective approach. This can be achieved through two methods: increasing the inoculum volume or its concentration. Larger inoculum volumes can be realized using high-density cryovials (e.g., 5 mL vials) or cryobags (e.g., 50 or 100 mL bags). Higher inoculum concentrations can be obtained using perfusion culture technology, which has been employed to prepare high-density cell banks (HD Cell Banks). Novel seed culture technologies have been established by combining HD cell banks, single-use technologies, and perfusion systems at the N-1 bioreactor stage [6][24]. This innovative approach reduces seed culture complexity by minimizing the number of intermediate scale-up steps and decreasing manual operations such as cleaning, assembly, and sterilization.

2.4.2 Concentrated Fed-Batch Culture

Concentrated fed-batch (CFB) cell culture employs perfusion technology combined with ultrafiltration modules to retain both cells and protein products. CFB enables flexible manufacturing by achieving high cell densities within limited volumes, allowing small-scale bioreactors to match the productivity of large-scale systems. Yang et al. [25] developed and applied CFB processes to two cell lines in laboratory-scale bioreactors. Compared to conventional fed-batch cul-

ture, CFB increased the yield of cell line A by 105% and cell line B by 70%, without significantly affecting product quality. Notably, the charge heterogeneity of cell line B was improved, demonstrating both process and product quality advantages of CFB.

2.4.3 Hybrid Culture Process

Hiller et al. [26] investigated a hybrid culture process that employed perfusion for the first four days to rapidly increase cell mass, followed by cessation of perfusion and daily addition of concentrated feed. Using five CHO-K1 cell lines, this approach achieved up to 2.5-fold higher productivity compared to conventional fed-batch culture. During the process, cell densities reached 60-80$\times10^{6}$ cells/mL with relatively low perfusion medium consumption (1-1.8 times the final bioreactor volume). The process utilized a novel perfusion control system enabling online monitoring and automatic regulation of perfusion rates, thereby achieving automation. Consequently, this hybrid process can enhance the performance of existing production lines.

3. Perfusion Culture Process Development

3.1 Process Control

Stable processes require proper control of medium addition and removal rates to maintain the desired perfusion rate. By monitoring viable cell concentration in real-time and regulating cell bleeding based on parameters such as stable, optimal cell-specific perfusion rate (CSPR) and perfusion rate, constant working volume and cell density can be maintained in the bioreactor. In the absence of online cell density control systems, semi-continuous cell bleeding is most commonly used to control cell mass. Karst et al. [19] implemented continuous cell bleeding using both online and offline control systems to maintain stable cell density; however, offline control levels can impact process performance and product quality, necessitating cautious application of this semi-continuous approach in commercial-scale manufacturing.

Volumetric productivity and product quality are two key performance indicators in biopharmaceutical cell culture processes. Yang et al. [27] optimized capacity utilization and production efficiency while maintaining or improving product quality through perfusion culture of three different CHO cell lines by increasing volumetric productivity. J. Rodriguez et al. [28] demonstrated that low-temperature perfusion culture for recombinant human interferon-β (β-IFN) production in CHO cells increased yield 3.5-fold and volumetric productivity 7-fold compared to batch culture, while reducing aggregates by 39% and enhancing bioactivity. Hiller et al. [26] found that short-term perfusion followed by conventional fed-batch culture with highly concentrated feed nearly doubled overall productivity compared to traditional fed-batch processes. Warikoo et al. [9] showed that an integrated system combining perfusion reactors with four-column periodic counter-current chromatography (PCC) for continuous protein

capture achieved substantially higher volumetric productivity than current perfusion or fed-batch processes.

3.2 Medium Screening

Medium development is one of the most critical aspects of cell culture process development and optimization, both because medium composition is paramount to process performance and because of safety considerations. Early cell culture media utilized animal-derived components [29], exposing patients to various risk factors. The concept of decoupled perfusion medium (i.e., separate inlet streams to better control specific nutrients and perfusion rates) has provided new insights for medium development. Lin et al. [30] developed an effective perfusion medium by mixing basal medium and concentrated feed from fed-batch processes at specific ratios. After optimizing the ratio and adjusting nutrient concentrations and osmolarity, the resulting medium required only half the perfusion rate to maintain cell densities of 30×10^{6} cells/mL. This approach provides a valuable reference for perfusion medium development.

3.3 Culture Scale Miniaturization

Microscale bioreactors represent another option for small-scale cultivation, offering online pH and dissolved oxygen (DO) monitoring [32]. To date, they have been primarily used for fed-batch culture, though some semi-continuous medium exchange experiments have been conducted. Gomez et al. [33] optimized fed-batch processes by studying 13 clones using semi-continuous shake tube operations and perfusion reactors. Recently, several commercial novel reactor systems with continuous medium exchange and cell retention capabilities have demonstrated productivity in fed-batch applications and may play important roles in perfusion process development, though more data are needed to fully evaluate their potential.

3.4 Critical Quality Attributes

The transition from batch to continuous production is driven by two factors: operational cost savings and, more importantly, improved target protein quality [4]. In discontinuous systems such as batch and fed-batch cultures, accumulated toxic substances and reaction by-products adversely affect product quality [3]. In perfusion culture processes, a stable culture environment can be maintained throughout the production period, and all kinetic parameters in the bioreactor —including those related to impurities or post-translational modifications—remain constant over time, resulting in consistent product generation without the cumulative effects observed in batch and fed-batch processes [4].

Gomez et al. [33] demonstrated that quality attributes such as galactosylation, afucosylation, and aggregation are comparable between shake tube and small-scale bioreactor models, while other attributes are more dependent on product residence time. For instance, deamidation, C-terminal lysine processing, and

clipping were found to differ in semi-continuous modes. Therefore, perfusion culture can enhance quality attributes by reducing product residence time in the medium.

4.1 Advantages of Perfusion Culture

Perfusion culture provides a stable, cell-friendly environment by continuously removing by-products and adding nutrients. Compared to batch and fed-batch processes, perfusion mode extends the duration of favorable culture conditions at high cell densities while reducing product residence time in the reactor, which benefits product quality and is essential for unstable products [35].

Furthermore, biotechnology companies must rapidly adjust production capacity to accommodate fluctuating market demands, while intensifying competition from biosimilar companies drives cost reduction [9][35]. Another advantage of perfusion culture over fed-batch processes is the ability to use smaller bioreactors, which reduces cleaning operations and allows replacement of stainless steel reactors with single-use systems due to smaller working volumes. Beyond biopharmaceutical production, perfusion culture can be used to generate high-density seed banks and cell banks or as a research tool for protein drug production. It can compensate for low cell-specific productivity by generating target proteins at high cell densities, thereby reducing manpower requirements for process development.

From a regulatory perspective, the FDA and other agencies maintain an open and supportive stance toward continuous manufacturing, recognizing continuous perfusion as a technology that enables stable and more efficient production [5][34][35]. Due to its versatility across product portfolios, several major domestic biopharmaceutical companies have begun transitioning from traditional fed-batch to perfusion culture to address limitations of conventional processes.

4.2 Limitations of Perfusion Culture

Perfusion culture also has limitations, presenting numerous challenges in technology development and aseptic processing. Additionally, the process generates multiple harvest batches that accumulate as numerous intermediate-volume collections requiring further processing [15].

Long-term perfusion culture at high cell densities significantly increases the risk of membrane fouling in most cell retention devices. Novel retention devices can effectively mitigate this issue. The alternating motion of ATF creates a scouring effect that helps prevent hollow fiber membrane clogging [16]. Xu et al. [31] optimized perfusion medium by adding pluronic polymers (poloxamers); low concentrations of these polymers facilitate product and host protein passage through cell retention devices while providing cell protection, thereby reducing membrane fouling risk.

Most perfusion media used in process development are custom-formulated, as

commercially available perfusion media remain limited, posing a challenge to perfusion culture development. Perfusion media typically contain richer nutrients than conventional basal media. Studies have shown that media prepared by mixing basal medium and concentrated feed at specific ratios can sustain high cell densities and viability [25]. Yang et al. [27] demonstrated that using two-fold concentrated medium in N-1 perfusion bioreactors can reduce perfusion rates while achieving higher cell growth rates, providing direction for perfusion medium development. Regarding cost control, Xu et al. [31] investigated productivity of the same cell line across different processes and found that perfusion culture medium costs could potentially be lower than fed-batch medium costs when bioreactor utilization is high.

Under stringent quality and regulatory requirements in biotechnology, the biopharmaceutical industry pursues minimal development time and cost control, inevitably leading to conservative adoption of new technologies. Typically, companies only implement incremental optimization measures, while more significant technological innovations become less common. Biotechnological innovation has shifted to become more product-centered rather than process-centered. Although these business drivers remain dominant, biotechnology companies now require flexibility to accommodate both high-volume and low-volume drug demands (niche or orphan drugs) within the same production line. Future biomanufacturing facilities are transitioning from large, single-product lines to smaller, more diverse, and flexible production lines that enable companies to adapt to evolving market demands [25]. Yang et al. [25] demonstrated that perfusion culture could enable small-scale equipment to replace large-scale equipment in terms of production capacity.

5. Conclusion

As an innovative technology, perfusion culture has significantly expanded our understanding of manufacturing processes. It addresses issues such as unstable protein quality or low expression levels, and the inability of fed-batch culture to ensure consistent batch-to-batch control, while eliminating unnecessary intermediate scale-up steps and simplifying the production process. However, this specific culture mode also faces challenges, including how to prevent contamination during long-term culture and how to verify consistency among "sub-batches" from harvest to purification—issues that become particularly critical during Investigational New Drug (IND) applications.

Although the key process development points discussed above may appear discrete, they are in fact highly interdependent. In practice, one must not consider only the impact of individual factors on outcomes; rather, multivariate analysis should be employed to fully examine relationships between different variables and their combined effects on product expression and quality. Therefore, achieving continuous perfusion culture processes for stable, uniform expression and production of high-quality proteins requires further investment of time and effort.

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