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Introduction to Continuous Manufacturing: Technology Landscape and Trends

The classic and largely predominant approach to bioprocessing, both upstream and downstream, remains batch processing, with manufacturing batch fluids essentially moving incrementally en mass as a bolus from one process step and set of equipment to the next. This assembly line-like, finish-one-step then move all the process fluids to the next, approach certainly works well but a number of technological advances and related trends are making continuous bioprocessing attractive. Continuous bioprocessing strategies are making advances and are being adopted or considered for many new drug bioprocesses being implemented. Meanwhile some established bioprocessing facilities are being retrofitted and upgraded for more continuous operations. Continuous upstream bioprocessing is actually not new, with fiber-based perfusion bioreactors widely used for classic fused-cell hybridoma culture, e.g., in the 1980s, when it was replaced by recombinant antibody manufacturing methods.

We can expect higher future adoption of bioprocessing by continuous methods[1,2]. Already, about a dozen or more marketed recombinant protein products are manufactured using perfusion or other continuous bioprocessing technologies. Leading adopters include Genzyme and Bayer. Most adoption of continuous bioprocessing has involved upstream processes, with continuous downstream purification tending to lag behind. Thus, it is currently common for new bioprocesses being implemented to combine continuous upstream processing with conventional batch purification. Continuous chromatography technologies, such as simulated moving bed (SMB) and periodic counter-current chromatography, are generally not yet ready yet for commercial-scale adoption. Regulatory barriers to continuous bioprocessing, such how to define lots, have been resolved, and continuous processing fits better than batch processing with automation, QbD and PAT. These aspects are making the benefits of continuous processing increasingly attractive to biopharma manufacturers.

Continuous upstream bioprocessing generally involves retaining production cells within the bioreactor at a fixed volume and fixed cell concentration on a continuous basis, such as for 30-90 days or even longer. The bioreactor fluid has a much higher cell concentration, with cells retained within the bioreactor by various methods.

The current leading method involves use of specialized filter-based equipment. Other methods for cell retention are done by centrifugation and use of capillary or other fiber-based and microcarrier reactors where cells self-attach to fiber or particle substrates.

There are many benefits to operating bioprocesses continuously rather than in batch mode, with many of these similar and complementing those of single-use and modular systems. These benefits include:

a) **Reduced costs**: Operating continuously allows use of much smaller-scale equipment, with a smaller volume bioreactor (and smaller sizes for most other equipment) operating over time resulting in as much product as much larger equipment operated in fed-batch mode. Besides smaller-scale equipment generally costing less, this allows much smaller facilities and equipment foot-print, with less space and utilities required, particularly when single-use systems are used.

b) **Increased productivity**: Because much of the bioprocessing equipment is operated continuously, there is little need for large transfer/storage vessels and no halts between processes. Bioprocessing thus tends to move much more smoothly. Much higher bioreactor cell densities can be attained, providing higher product yield and concentration. Also, the number of bioprocessing staff required is decreased, and their work at large scale is less physically demanding.

c) **Improved quality**: Biological molecules are naturally produced continuously, and compared to batch culture, continuous culture tends to be more controllable, less intense and stressful, including less shear and media nutrient levels kept constant. Product variability, e.g., later culture stage-related loss of cell viability or altered glycosylation, is reduced, with continuous bioprocessing inherently more consistent and robust. Problems associated with proteolytic or other degradation over time in bioreactors and other vessels can be avoided or minimized. And if any problems do occur, only part, not the entire, production run likely needs be rejected.

d) **Increased flexibility**: Continuous manufacture enables more adaptability and efficient facility utilization, similar to the advantages of single-use devices. Bioprocessing becomes much more portable, and facilities more clonable. Couple this with the trend for adoption of modular bioprocessing systems, multiple smaller continuous bioprocess lines in smaller facilities worldwide, and we expect this approach will be increasingly adopted for commercial manufacturing.

The BioPlan 10th Annual Report and Survey of the Biopharmaceutical Manufacturing evaluates key trends and aspects of the bioprocessing industry. We surveyed the attitudes of 300 industry professionals towards perfusion and continuous processing in 2013 [3,4]. Attitudes are common with relatively new bioprocessing technologies. Overall, respondents saw more problems associated with perfusion/continuous vs. fed-batch processing. "Process complexity" was the primary concern, cited by 69% (% indicating this factor either "much bigger" or a "somewhat bigger" concern), followed closely by "Process development control challenges" noted by 64.7%. Other issues included "Contamination risk" at 58.6% and "ability to scale-up" at 54.3%. In comparison, for the same aspects, concerns over batch fed processes were noted by very few (single-digit percentages) respondents. Much of this perception will likely change as the industry is increasingly exposed to the successful application of continuous technologies in clinical and commercial scale bioproduction.

'When specifying bioreactor types for a new [Clinical] or [Commercial] scale biologics facility, how likely are you to specify" % "very likely" or "likely"

Fig.1: Likelihood of Implementing Bioreactor, by Type
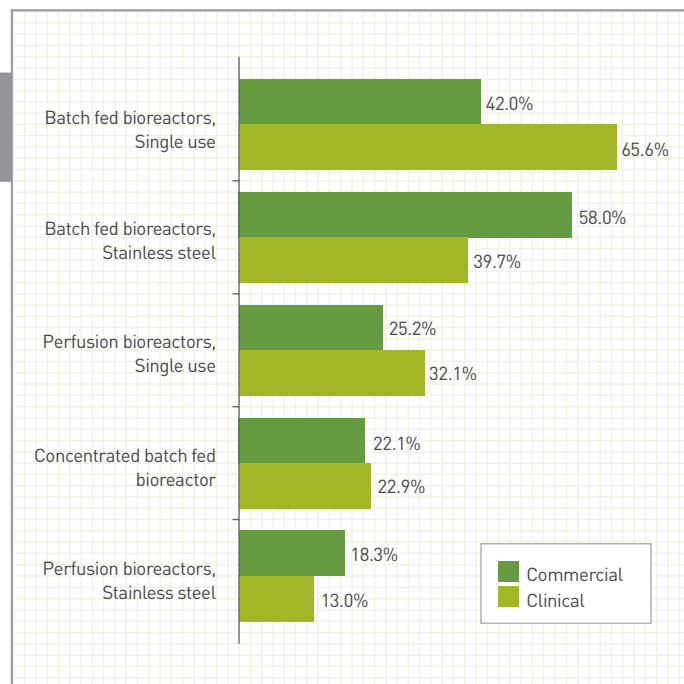
Source: 10th Annual Report and Survey, Biopharmaceutical Manufacturing, April 2013, BioPlan Associates, Inc. Rockville, MD

In fact, continuous processing equipment manufacturers and users rather uniformly report that many of these problems have been resolved with application of current technologies, including single-use equipment. Perfusion/continuous processing is now generally significantly less complex, less prone to contamination and more readily scalable than fed-batch methods. Industry perceptions of perfusion/continuous vs. fed-batch are lagging, and likely reflect a lack of direct exposure or experience with the technology. When those surveyed were asked what types of bioreactor they would implement for a new facility coming online in 2 years, as expected, over two-thirds cited batch-fed single use bioreactors, while 32% and 25% cited single use perfusion bioreactors at clinical and commercial scales, respectively.

BioPlan Associates expects increased and rapid adoption of continuous bioprocessing at all scales, including commercial manufacture. The imperatives of cost-savings, flexibility and product quality will increasingly drive the industry to explore continuous processing. This, in turn, will expand the industry's current knowledge and experience base, when making major changes in manufacturing platforms. Particularly, as perfusion and other continuous bioprocessing technologies are improved and increasingly adapted for single-use equipment and modular systems, adoption will further accelerate. Many upcoming continuous bioprocessing technologies are actually very novel. For example, a single 5 L bioreactor currently in development will be able to manufacture the same quantity of product, often at better quality, comparable to a 5,000 L over the same time period using the same amount of media [1]. Case studies and other reports of such performance will further promote rapid adoption.

We predict increasingly rapid adoption of single-use systems for the majority of new commercial manufacturing facilities over the next 5 years, and we expect continuous bioprocessing, particularly for upstream processing, to follow a similar trajectory. Use of these products is likely to further increase with hybrid systems that use bolt-on-type technology, that retrofit components unit operations for existing systems. Other conventional technologies, such as centrifugation, will also seen increasing adoption in coming years. Potentially revolutionary capillary fiber perfusion bioreactors and other new technologies, including those for downstream processing, will be likely coming online and be more widely adopted for commercial manufacture over the next 10 years.

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Jerry Shevitz, Ph.D. received his degree in biochemistry from CUNY, New York, N.Y., subsequently focusing on cell biology and Immunology. Jerry has an extensive background, spanning about three decades, in the biomanufacturing industry, from small scale research to large scale process development, with companies involved in drug development and also with equipment suppliers. Jerry is a productive inventor always looking for improved solutions and new approaches. He is President of the company and heads the Refine research department.

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A Brief History of Perfusion Biomanufacturing
How High-Concentration Cultures Will Characterize the Factory of the Future

Today's renewed interest in perfusion culture is due to an increased awareness of its advantages, some general improvement in equipment reliability, and a broadening of operational skills in the biomanufacturing industry. Some misperceptions persist, however, according to a 2011 review by Eric Langer.[1] Our view here of the history of perfusion and fed-batch processes includes some discussion of technological process improvements and challenges that the bioprocess industry faces.

A team of authors at Serono in Switzerland wrote in 2003:
The major advantage of the perfusion mode is high cell number and high productivity in a relatively small-size bioreactor as compared with batch/fed-batch. In order to sustain high cell number and productivity, there are needs to feed medium during the cell propagation phase and the production phase. In contrast to batch and fed-batch processes, where there is no metabolites removal, in continuous processes medium is perfused at dilution rates exceeding the cellular growth rate. For this, a good separation device is needed to retain cells in the bioreactor.[2]

Many cell retention devices perform well, to a greater or lesser degree, at small scale, including gravity-based cell settlers, spin filters, centrifuges, cross-flow filters, alternating tangential-flow filters, vortex-flow filters, acoustic settlers (sonoperfusion), and hydrocyclones. All are described well in the 2003 paper mentioned above. But only a few types are reliable at larger scales and scalable enough for bioindustrial use.

Here we compare the ATF System from Refine Technology with spin filters, cell settlers, and centrifuges. We am not including other technologies here because of scalability limitations and a lack of proven market acceptance.

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Perfusion's Early Potential

The advantages of using perfusion for enhancing production of cell-derived products were realized in the late 1980s and early 1990s. In those early days of the modern biotechnology industry, production cell lines were not fully developed, and their product expression was very small — from a few micrograms to a few hundred milligrams per liter in batch or fed-batch. Attainable cell concentrations were only a few million per milliliter.

Spin Filters: Perfusion offered a way to derive more product from such low producers. It was well known that perfusion could increase cell concentration by as much as an order of magnitude [3]. The spin filter was the most common perfusion device used; it was the best cell-separating device available at the time, supported by reputable equipment manufacturers.

Spin filters remain in use at a few sites but have been largely phased out, largely because of their limited scale-up potential and unreliability: When a bioreactor's volume scales up by the cube of its radius, the surface area of its spin filter screen scales by the square of its radius. An internal spin filter can take up a significant portion of production space within a vessel, and once its screen fouls, the run is terminated. An external production spin filter may solve this shortcoming, but it has drawbacks related to cost, maintenance and sterilization difficulties.

A more important factor behind the lackluster acceptance of perfusion in those early years was the rapid evolution of cell biology. New, more productive expression systems and improved media development permitted large increases in culture productivity; product concentrations were increasing from several hundred milligrams to about a gram per liter. Production needs could, therefore, be achieved with the well-understood fermentation technologies, batch and fed-batch. Scale up was accomplished simply by moving to bigger vessels.

The success of batch and, more important, fed-batch, not only inhibited the wider acceptance of spin filters, but also of other evolving cell-separation technologies. The difficulties associated with spin-filter operations and the undeveloped state of new perfusion technologies stigmatized the process. The dominance of fed-batch continued well into the next decade.

However, despite the dominance of fed-batch as an industry standard, perfusion continued to be championed. Perfusion offered an excellent solution for production with unstable proteins that could not remain in the toxic environment of an ever-deteriorating fed-batch culture. With perfusion, such products could be removed rapidly from a vessel and stored appropriately to preserve their stability. Many people chose perfusion to bypass constraints of space and cost factors. Furthermore, as culture productivity increased, and although it greatly benefited fed-batch processes, perfusion promised even greater output from a continuous culture.

Perfusion performed at CMC Biologics

So the use of perfusion never died; in fact, as the use of spin-filters declined, other cell separation devices slowly emerged. Those were based on filtration, gravity settling, and centrifugation. Continued development of numerous products that held out the promise of commercialization provided the driving force to experiment with new culture technologies. Occasionally a perfusion process, was scaled to commercial production.

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High cell concentrations are a game-changer: From the early 2000s and particularly in the past few years another critical transition in biopharmaceutical manufacturing occurred. Further advancements in development of cell lines, expression systems, and media formulations resulted in an impressive ability to grow cells to very high concentrations and achieve product concentrations previously inconceivable. Using fed-batch as a reference, in the mid 1990s attainable cell concentrations were about 5×10^6 cells/mL, with record product concentrations of 1–2 g/L; today those are greater than 15×10^6 cells/mL, with product concentrations of up to 10 g/L. Although those concentrations are still not typical, they indicate where the field is heading. Those results are amplified by the use of perfusion, through which substantially higher cell concentrations and product output can be achieved. [4, 5]

Perfusion Returns to Manufacturing

A general lack of manufacturing capacity forecast at the beginning of this century was overcome through both biological innovation and engineering construction. Today's overcapacity places most of the available space in the hands of relatively few companies. Even as some large biofacilities are mothballed, newer companies build modern facilities based on the latest technologies. Few organizations would now consider building a new, multiple–20,000-L bioreactor facility. Rising competition in the healthcare sector, whether through generics/ biosimilars or multiple drugs with the same indication, requires the vast majority of biopharmaceutical products to be more easily produced in smaller and more flexible plants — even in multiple locations. New ultrahigh-density cell culture processes such as concentrated fed-batch and concentrated perfusion are well suited to this new manufacturing environment and facilitate a shift toward single-use technologies. That helps companies reduce both risk and capital investment, allowing them to delay making major facility decisions.

So the face of biomanufacturing today is very different from that of just a decade ago. Nearly everyone uses perfusion in some way — from large biopharmaceutical companies such as Pfizer, Medarex, and Genentech [6–8] to small biotech and novel vaccine manufacturers such as Shire and Crucell [9, 10]. In addition, today, contract manufacturers, such as Gallus Biopharmaceuticals and Rentschler, run several commercial perfusion processes allowing companies a choice and

the option of contracting out a continuous biomanufacturing platform. Outside the established biomanufacturing infrastructure, biosimilar and other relatively new biological manufacturers such as Biocon and Kanghong Biopharma are also looking favorably on the perfusion model because of its associated cost efficiency. Perfusion is back.

Simplicity and reliability have long been key factors to consider in biologics production, especially where manufacturing involves high-value products in a large-batch environment. The industry is now being challenged as it moves forward to realize the much–touted "factory of the future," which will incorporate several platform technologies. One such technology is certainly the adoption of disposables throughout production facilities.

Microcarrier-based perfusion in a single-use ATMI bioreactor

Perfusion is a broad term, which many people may still view unfavorably. Although many, in fact, use perfusion at some level, not everyone admits to it — nor to how they do it, nor how often. Companies are experimenting with perfusion to solve challenges or implement novel solutions at many process stages: high density, large-volume cell banking [11]; seed expansion [8]; n–1 perfusion [12]; and of course

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final production reactors[13]. Perfusion has evolved too: It is no longer solely a two- or three-month process, but can be as short as a three-day boost to a standard fed-batch process. Perfusion has become a specialist operation. Implementation depends on the nature of different facilities, cell lines, processes, and products — as well as each company's own operating philosophy. Success depends on many factors, not least of which is a company's choice of perfusion system. But one challenge — that of producing a reliable cell-retention device — may have been solved to a great degree by a relatively new hollow-fiber perfusion device.

Case Study

The ATF System (Fig. 1) offers nearly linear scale-up for simplicity of operation and validation. Generally, conventional filtration systems will fail rapidly when used to separate media from a complex suspension of a cell culture with a high bioburden. By contrast, this particular system, due to its flow dynamics, has an inherent self-cleaning ability to allow its range of filter materials and pore sizes to perform significantly longer than might otherwise be expected.

Fig. 1: The ATF System
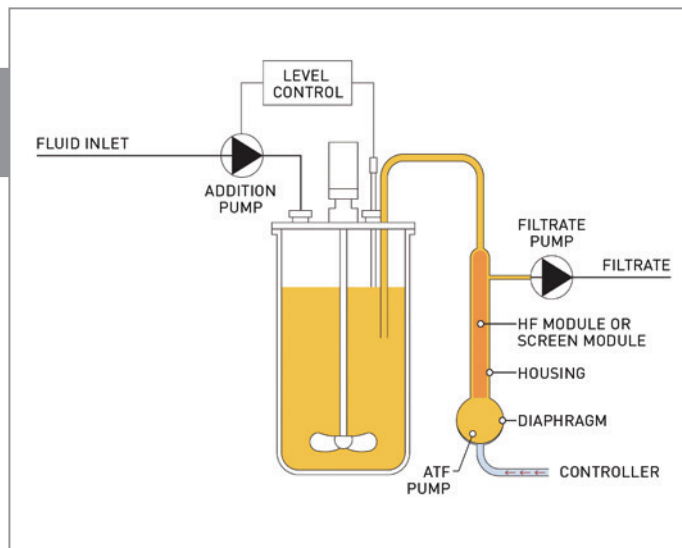
A standard hollow-fiber module is used to separate cells and product. However, unlike systems that recirculate a culture through a filter in one direction, the alternating tangential-flow action constantly cleans the fibers every five to ten seconds with a backflush action. With only a single connection to the bioreactor, cells and media enter and leave the ATF System, flowing reversibly through the hollow fibers. Flow is controlled by the diaphragm moving up and down in the ATF System's pump. This generates a rapid low-shear flow between vessel and pump, ensuring rapid exchange and prompt return of cells to the reactor and minimizing their residence outside the bioreactor. The choice of pore size for the hollow fiber determines what elements are retained and which ones pass through to the permeate.

From Research to Manufacturing — the Scale-Up Challenge:

For companies requiring increased protein production in preclinical work, many perfusion technologies can quickly deliver.

One common approach is to choose a small-scale cell-retention device that offers a high degree of confidence for scaling to a commercial manufacturing process. Scaling up a bioreactor introduces its own issues, so engineers don't want perfusion equipment to add further complications. Several technologies have been used at large scale, and each system brings its own limitations. For example, well-known spin-filter technology, previously discussed, uses a two-dimensional screen to retain the cells. Limitations of the system (whether internal or external) arise during scale up and at elevated cell concentrations when rapid feed rates are required. Consequently, to reduce risks of screen blockage, the process duration must be shortened or the culture maintained at low cell concentration to prevent excessive accumulation of cell debris on the screen. The latter is usually what occurs.

Different but familiar problems occur with inclined or gravimetric settlers. Cells spend significant time in an external, suboptimal environment within the settler (particularly) as the size of a system is increased. Additionally, as a system is increased, when greater perfusion rates are required, raising recirculation flow rates can lead to inefficient cell separation and significant cell loss, which lowers output and increases costs.

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Centrifuges have been scaled up successfully for several perfusion processes, often to very high flow rates. However, the high level of fine-tuning required to maintain the reproduceability of such systems — particularly during scale up — as well as their cost greatly discourage their use.

Despite those issues, each cell-retention device has a solid following among a number of companies. Skilled and experienced individuals maintain such systems. They assess and improve scale-up and scale-down performance.

An Xcellerex bioreactor used in an intensified perfusion process with ATF System equipment

For companies that require simpler systems that can be operated by a nonspecialist or that do not want to devote years to building those requisite skills, the ATF System can provide a robustly scalable process platform for most cell lines. Laboratory-scale devices are run as standard to produce the same conditions and flows that commercial scale devices will use. Two key parameters to keep constant are the filtrate flow ratio and the flow through each individual hollow fiber. Other parameters that would normally require attention — e.g., filter surface area and residence time — are factored into the equipment configuration design to limit variability potential. Scale-up is therefore straightforward to help teams build their confidence and experience rapidly. Additionally, unlike the older systems, a failure in the ATF System does not mean failure of the run. The perfusion device can be easily exchanged with another in a sterile way to continue the process. Bioreactor issues actually come to the fore: Can a large-scale bioreactor handle the oxygen demands of a cell concentration that is about 10 times higher than usual?

A Factory of the (Near) Future

A stable cell line is a prerequisite for a perfusion process if it is intended to produce a high-quality product for an extended time. Considering the state of biological manufacturing today and industry trends of the past two decades, some features of the factory of the future can be anticipated:

A Continued Move Toward Single Use: Innovations in disposable bioreactor designs have moved the industry toward their increased use. That trend is reflected by the large number of companies that are currently supplying single use Bioreactors (SUBs). Innovative SUBs from sub-one liter to 2,000 L are readily available today. Along with SUBs, significant improvements have been made in processing equipment, sensors, and other components, all with disposability in mind.

A Shortened Bioreactor Train: The ability to generate high-cell-concentration cultures combined with the ability to freeze large volumes of such cultures has made it possible to create high-volume cell banks. A single sample can be used to inoculate a relatively large bioreactor directly, eliminating multiple steps, saving time, and greatly increasing reliability.[14]

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Simplified Product Stream: Generating a filtered product stream by filtration perfusion can shorten the steps between vessel and column.

Concentrated Perfusion: Although 1 g/L/day is routinely achievable today using concentrated perfusion, 2-3 g/L/day[15] has been reported, and 5 g/L/day can be regarded as the next step. The volumetric productivity of concentrated perfusion means that at 5 g/L/day, one 500-L reactor would produce 2.5 kg of protein every day, and over 500 kg/year.

If these goals are achieved in the foreseeable future, there is little reason for even a high-dose blockbuster to be manufactured in anything larger than a 500-L vessel, whereas most other products could be handled with current laboratory-scale equipment. The future size of the factory, for upstream processes at least, looks very small indeed.

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03

Bioreactor Configuration and Operation

Through the higher cell densities and titers achieved in concentrated fed-batch and perfusion cultures typically smaller culture volumes and bioreactor scales are required to produce the therapeutic protein or antibody. This is a great advantage as the footprint of a production facility can be reduced and scale-up issues are mitigated due to the marginal scale-up factor between clinical and commercial production. Intensified cell culture processes are especially beneficial in the context of single-use facilities as they provide production capacities at 1000L scale that in the past where only achievable with ten times larger bioreactors.[1,2] Furthermore, modern high-end cell culture processes aim to maintain the cells in a defined metabolic state in order to ensure stable product quality through controlling protein folding and glycosylation. In this case, the main aim is not necessarily to reach very high cell densities, but to ensure a steady state of nutrients and metabolites in the bioreactor.

How to perform concentrated fed-batch or perfusion operation

After inoculation of the bioreactor and an initial 1 – 2 day batch growth phase, the removal of cell free supernatant e.g. with the Refine ATF System is started at a constant harvest flow rate. At the same time, the culture is replenished with fresh medium. When applying single-use bioreactors such as the Biostat® STR, the addition is controlled via a feed pump that receives a signal from load cells or a platform balance maintaining a defined bioreactor weight. As the cell density grows and the nutrient consumption and metabolite formation increases, the harvest rate is subsequently increased to maintain a certain exchange rate of fresh medium per cell or alternatively a given medium exchange rate per day.[3] On-line biomass measurement, e.g. with the BioPAT® ViaMass probe that will soon be available for single-use Biostat®STR and RM bags, provides an automated option to control the perfusion rate based on cell density. Using at-line glucose and lactate measurement, e.g. with the BioPAT®Trace, an additional concentrated feed can be applied to control the glucose concentration.

Figure 1 provides a schematic depiction of a typical concentrated perfusion or fed-batch set-up based on the Biostat® STR.

Fig. 1: Set-up of a concentrated fed-batch using the single-use bioreactor Biostat® STR
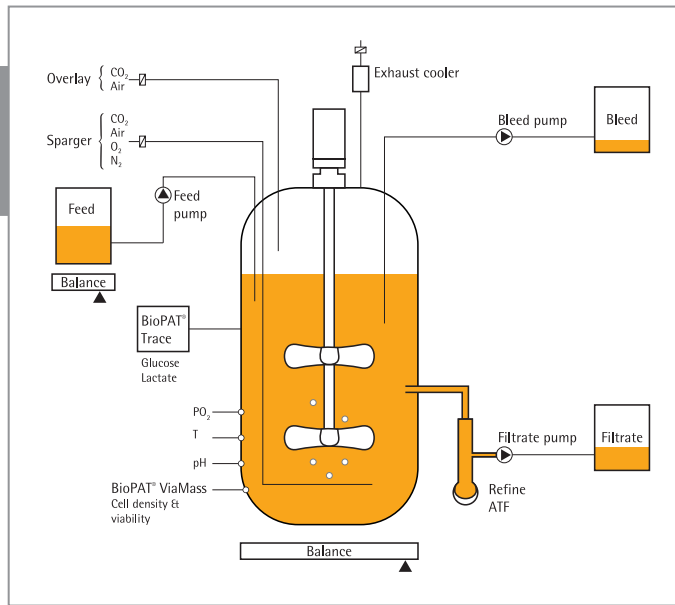
Key considerations

Typical perfusion rates are in the range of 1 – 2 bioreactor volumes per day. Applying a small cell bleed stream enables the establishment of a defined cell growth rate and by that a high viability can be maintained which in turn mitigates clogging of the cell retention device.[4] Dependent on the pore size or cut-off of the cell retention membrane, either the product is recovered in the cell free harvest (concentrated perfusion) or in the bioreactor content (concentrated fed-batch). As most antibodies are rather stable, concentrated fed-batch with accumulation of the product in the bioreactor is a simple and straight forward approach to increase space time yields of a given facility. Concentrated perfusion is the method of choice for recombinant proteins that in many cases are prone to degradation or might show feedback inhibition and should therefore be removed from the cell culture into a chilled harvest tank and subsequently purified.

Using Sartorius stirred tank single-use bioreactors in combination with different sizes of the Refine ATF System, concentrated fed-batch and perfusion processes can be developed at the 2L bench scale, e.g. using the Univessel® single-use in combination with our Biostat® B or B-DCU controller and subsequently scaled to 500L to 1000L scale in the Biostat® STR. At 500L and 1000L scale, the ATF System filter modules might be connected via side ports of the single use bioreactor bag using up to two 1″ sterile connectors and operated in an external loop of the bioreactor. It is critical that this external loop is as short as possible to avoid that the cell culture is exposed to uncontrolled conditions, e.g. different temperature and potential oxygen limitations.

Single-use bioreactor configurations suitable for intensified cell cultures

Key to successful concentrated fed-batch and perfusion operation is an efficient aeration system that provides kla values above 10 - 15 h^{-1} to supply the culture with sufficient oxygen (Fig. 2). At the same time, excessive carbon dioxide is formed in the intensified culture which needs to be removed to avoid any inhibitory effect on productivity or even product quality.
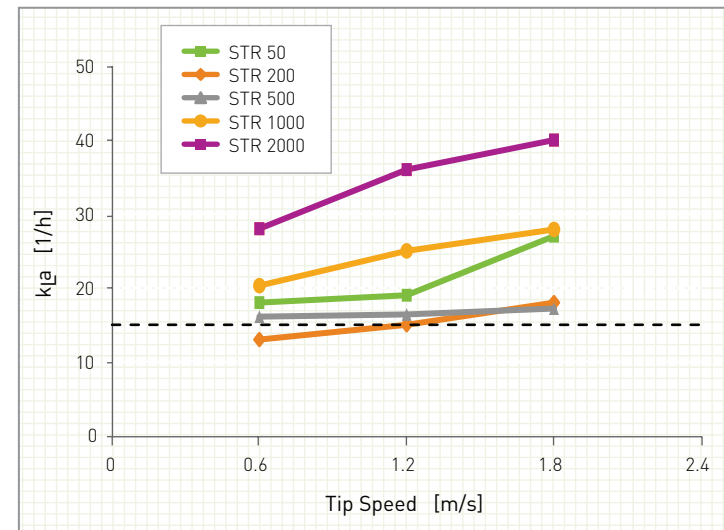

Fig. 2: kla values determined in different single-use bioreactor bag volumes of the Biostat® STR.

kla values determined in different single-use bioreactor bag volumes of the Biostat® STR, equipped with 2x3blade segment impellers, using the gassing out method in phosphate buffered saline, aeration rate 0,1vvm, 150μm holes of Combisparger, temperature 25°C.

This can be achieved with the Combisparger that microsparges compressed air or pure oxygen through defined 150µm holes and provides a stripping gas flow through 0,8mm holes at the same time (Fig. 3). This single-use sparger design emulates a successful aeration strategy applied since many years in conventional stainless steel bioreactors.
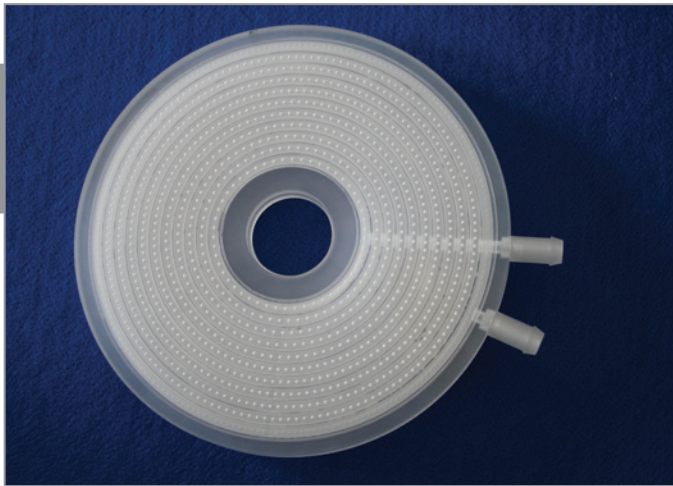

Fig. 3: Combisparger with 150µm defined micro-holes and 0,8mm holes.

A problem that should not be underestimated is excessive aerosol formation in the exhaust gas due to the high gas flow rates and the high protein content in the concentrated cell cultures. A specifically developed single-use exhaust cooler design based on the well-known principle of plate heat exchangers (Fig. 4) mitigates the risk of blocked filters and increases process reliability dramatically. Additional safety locks in the bioreactor control software prevent bioreactor overflow in case of clogging of the cell retention device. As a worst case safety lock, all feed pumps and gas flows are interrupted if the pressure in the bioreactor exceeds the maximum defined operating pressure.

Modern single-use bioreactor designs such as of the Biostat® STR allow advanced, intensified cultivation strategies whilst providing tools to mitigate operational risks associated to a complex bioprocessing strategy and thus enabling robust single-use production for clinical trials and commercial drug manufacturing.

Fig. 4: Exhaust cooler

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Véronique Chotteau has more than 25 years of experience in mammalian cell culture cultivation (suspension and adherent) and more than 10 years of experience in biopharmaceutical industry including different responsibilities as project manager, pilot plant head, expert in animal cell culture development (small, pilot and commercial size/GMP), business development support. Véronique joined Pharmacia Upjohn, Stockholm, (nowadays Swedish Orphan Biovitrum) in 1996. She worked with the process development of factor VIII Refacto, other perfusion processes as well as fed-batch processes. She left the industry in 2008 when she was offered to take over the group of Cell Technology, Dept. Industrial Biotechnology/Health, School of Technology, Royal Institute of Technology (KTH), Sweden. The activities of her group are focused on established cell-based processes (perfusion, metabolic flux analysis modeling) and stem cell bioprocessing.

04

How to Develop a Perfusion Process

1. Introduction

In a continuous process, the culture medium is continuously renewed by removal of conditioned medium and feeding of fresh medium while the cells are totally or partially retained in the bioreactor by a cell separation device. The volume of fresh medium is identical to the one of spent medium, which is preferably cell-free.

The process development can be divided in two main parts, the selection/**optimization** of the parameters and features of the **perfusion process** (excluding the cell separation device) and the **development of the cell separation process** itself. The selection of the culture length is made from information of both parts supplemented with Cost of Goods (COGS) and failure risk considerations.

The efforts of perfusion process development may vary depending on the purpose of the process: in several cases where the production yield is not critical, the efforts can be limited while more efforts might be needed when developing a commercial production process where the production yield and the product quality are highly important, and likely even more efforts will be needed if the product of interest (POI) is unstable or sensitive.

Limited efforts are required in the case of production for research purposes (i.e. case where the production yield is less important than the time needed to obtain the target product), for the production of cells aimed at cell banking or for inoculation of a larger bioreactor. In these cases, the accent is put on cell healthiness, i.e. high viability, and increased cell density while the cell stability is maintained. In the case of cell banking or cell seed manufacturing, high viability is required both for the success of the next operation step, respectively cell cryopreservation and seeding of larger bioreactor, and to avoid cell selection by cell death, which could result in cell population shift.

For the case of production for research purposes, often the cell lines are rapidly obtained but sub-optimal, i.e. with low cellular productivity. In that case working with a high cell density can compensate the low cell specific productivity while a high viability gives some insurance that the product quality is sufficient – however this is of course depending on the produced molecule.

2. Systems for development of perfusion processes

Generally speaking, the main trends observed in batch culture will remain true in perfusion culture, e.g. a favorable effect of a plant hydrolysate on the POI production observed in batch culture will most likely be confirmed when applied in perfusion process. It is advisable to confirm (and possibly refine) the observations made in batch culture in perfusion system before their application in a process.

2.1. Screening model

Small vessel cultivation systems aimed at screening larger numbers of conditions can be used for a pre-determination of parameters, followed by confirmation or refining at bioreactor scale. Typically, medium selection and effect of medium components can be screened saving labor and time.

A pseudo-perfusion process (also called semi-perfusion or quasi-perfusion), using shake flasks, spinners or 50 mL tubes with vented caps can be used to simulate perfusion. Daily medium renewal is operated manually: the culture is centrifuged, the supernatant is discarded partially or totally and the cells are re-suspended in fresh medium.

A main difference between the pseudo-perfusion system and perfusion is the residence time of the components, which is asymptotically evolving to the input value in the latter case. For instance, in a perfusion it takes 3 days at perfusion rate 1 reactor volume/day (RV/day) for a complete medium renewal since the fresh medium is constantly diluted in the culture (see Fig. 1). Contrary, in a pseudo-perfusion, the entire medium volume is renewed at once for the same apparent rate of 1 RV/day. Due to this difference, a partial medium renewal is sometimes adopted instead of complete medium renewal.
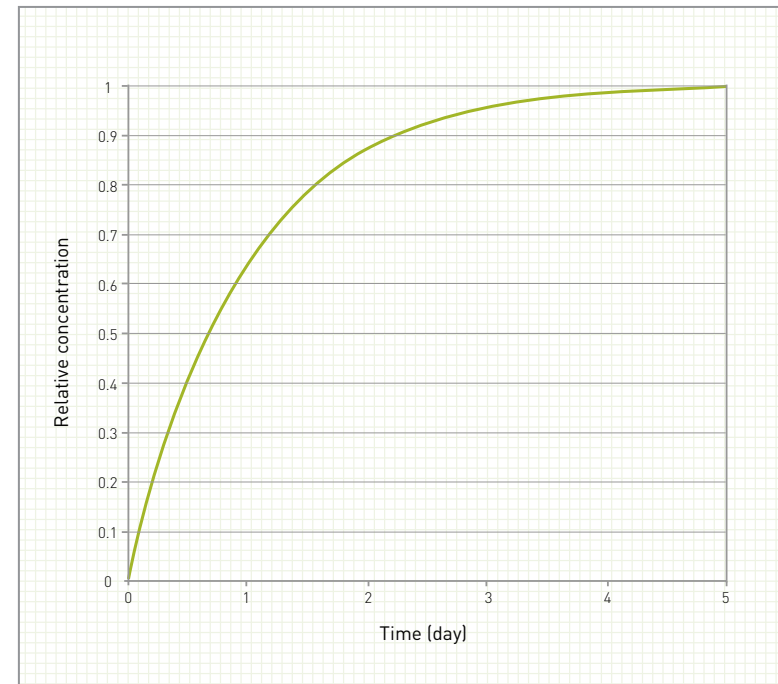

Fig. 1

Evolution with time of the relative concentration of a new component present at concentration 1 in the fresh medium of a perfusion process operated in bioreactor at 1 RV/day perfusion rate

2.2 Bioreactor and scale-down model

Bioreactor systems are used for the development of the perfusion processes and most of the parameters can easily be studied in scaled-down models. Exceptions are parameters such as the shear stress and the deleterious effect of bubble/gassing, for which the scaled-down study is more challenging. The study of the cell separation device itself has to take into account the limitations of the targeted large scale for parameters like the liquid flows or the power.

3. Development of the perfusion process

3.1 Medium selection

A culture medium needs to include all the necessary components to sustain the cell growth and production of POI, e.g. in case metal(s) or a vitamin are

crucial for the POI activity. Nowadays serum-free and chemically defined media provide not only these necessary components but moreover give enhanced cell growth, cell survival and/or production from additional components and optimized formulation.

A base medium can be advantageously supplemented with a feed concentrate, which has been developed for fed-batch process, to improve the POI production for instance. Starting the development of a perfusion process can be initiated by the evaluation of 5 to 10 commercial media in a batch shake flask productivity test study leading to the selection of 2 to 3 base media. Supplementation of these with different feed concentrates can then be studied. From this study a base medium, potentially supplemented with feed concentrate, can be selected and tested in perfusion mode.

Subsequent to this, the medium can be further refined/optimized if it is necessary to improve the POI cell specific productivity for the goal of the process. Another aspect is that since perfusion mode requires large volumes of liquid handling, minimizing the perfusion rate without compromising the process performances can be desirable. This can be achieved by tuning the medium composition and use of concentrated media (Konstantinov et al. 2006; Ozturk 1996; Runstadler 1992).

3.2 Perfusion rate strategy
Two main strategies can be distinguished to determine the perfusion rate: either based on the cell density or based on the availability of a main substrate in the culture. Sometimes, it is even desirable to increase the perfusion rate to reduce the by-product accumulation.

3.2.1 Perfusion rate strategy based on CSPR
An established strategy is to adjust the perfusion rate as a linear function of the cell density (Ozturk 1996; Konstantinov et al. 2006; Clincke et al. 2013b), i.e. to apply a cell specific perfusion rate, CSPR, where   CSPR = perfusion rate / cell density or D / cell density.

This allows avoiding the depletion of component(s) in the culture and has been demonstrated to sustain up to 200 x 10^6 cells/mL (Clincke et al. 2013b). In order to save medium, identifying the minimal CSPR (CSPR_min) is critical. A method to select CSPR_min is:

- inoculate the bioreactor at cell density 0.3 to 1 x 10^6 cells/mL, initiate the culture in batch mode and start the perfusion at D = 1 RV/day when the cell density has reached 2 to 3 x 10^6 cells/mL – importantly start the perfusion while the cells are still in exponential growth phase
- allow the cells to grow exponentially until e.g. 20 x 10^6 cells/mL, by daily monitoring the growth rate, while increasing D to 2 RV/day (or higher) in case the growth would slow down
- establish a culture around 20 x 10^6 cells/mL of exponentially growing cells by performing daily cell bleeds compensating for the cell growth – this culture is an excellent system to test various parameters like CSPR, pH, etc.
- in the culture stabilized at 20 x 10^6 cells/mL, identify the CSPR_min for the given cell line and medium with the following steps applied at 1 to 3 days intervals (duration required to observe the effects of an implemented modification):

  increase D of 0.5 RV/day step and go to either i) or ii) depending of the outcome
  → i) if the growth is increased (by increasing D), the actual CSPR is too low and D has to be increased (of e.g. 0.5 RV/day step). Repeat increasing D by 0.5 RV/day steps until further increase of D does not result in improved growth. The next to last D gives CSPR_min.
  → ii) if the growth is not increased (by increasing D), CSPR_min is not higher than CSPR in use and is possibly lower. Test to reduce D by 0.2 RV/day step and observe if the growth remains (or not) unchanged. In the positive, continue to decrease D; in the negative the next to last D gives CSPR_min.

Notice that after a slower growth has been observed, it requires some time (at least 3 days) for the system to recover from depletion (depending how severe the depletion was).

It is recommended to control the feed of glucose and glutamine separately, at a stoichiometric rate and to maintain their concentration at low levels when applying a CSPR during the process development phase. As a matter of fact, the need of these substrates can be different from the need of the other medium components. When the process is established, these substrates can advantageously be delivered together in the fresh medium formulation.

3.2.2 Perfusion rate strategy based on main substrate measurement

Control of the perfusion rate can be based on a main substrate like glucose (Dowd et al. 2001). Glucose is present at a selected concentration in the medium. From daily glucose concentration measurement, the perfusion rate is increased or decreased in order to maintain the glucose concentration constant in the culture. This can be based on daily manual glucose concentration measurement or on a more sophisticated on-line measurement of glucose.

3.3 Removal of toxic by-products

Ammonia and lactate are known for their negative effect on the cell growth and productivity. High ammonia concentrations are also reported to affect the glycosylation profile (Goochee et al. 1991; Jenkins et al. 1996). Using a dialysis system with 10 kDa cut-off (Buntemeyer et al. 1992) showed that spent medium could be re-used however this is not today an industrial practice. They also showed that other (un-identified) low molecular weight components than lactate and ammonia had a toxic effect.

In case the lactate or ammonia concentrations are reaching unfavorable levels, the perfusion rate can be increased to remove these by-products. A graphical representation of the effect of lactate or ammonia concentrations on the growth rate or the cell specific production rate can provide guidelines for the selection of limits of these by-products in the process.

3.4 Cell density – target, monitoring and control

Several strategies to perform a perfusion process can be adopted.

- **Stable cell density with growing cells**: Maintaining the cell viability as high as possible and the cells in growing stage is one of the main strategies used in perfusion field. After a culture period of increasing the cell density to a target level, the cell density is maintained stable at this level in a system where cell removal (automatically or manually operated) is performed at a rate compensating the cell growth (Konstantinov et al. 2006). Industrial processes are operated on this principle for months.

- **Increased cell density**: Another strategy based on growing cells is to increase the cell density until a physical limitation of the cell density itself or the equipment is reached – or close to be reached - (Clincke et al. 2013a; Clincke et al. 2013b).

- **Stable cell density with arrested cells**: A third strategy consists of a first culture period of increasing the cell density to a target level, then to slow down or completely arrest the cell growth, which is known to be potentially associated with a higher cell specific productivity in a cell specific way.

3.4.1 Inoculation cell density

Thanks to the medium renewal applied as soon as the cells have reached a couple of millions cells/mL (see Section 3.2.1 for an example), the inoculation cell density has not the same major impact as in a fed-batch process. The culture can be initiated as a batch culture in the conditions mimicking shake flask scale. The perfusion is then started when the cells are still in exponential growth phase. A higher inoculation cell density allows shorting down the time required to achieve the target cell density. An option in this latter case is to start the perfusion the same day as the inoculation.

3.4.2 Selection/optimization of the cell density

Two decades ago, the cell density in perfusion reached a few millions cells/mL in many cases. A standard in industry today is to target around 20×10^6 cells/mL but there is a trend towards much higher cell densities where the benefit of perfusion can be fully exploited. It is probable that today many industrial processes are targeting 50 to 80×10^6 cells/mL (Clincke et al. 2013a, Johnson T 2013).

3.4.3 On-line cell measurement

Besides manual sampling, the cell density can also be measured on-line based on the dielectric properties of the cell, i.e. permittivity and/or capacitance, by commercial probes. Recently in-situ microscope technology has also been developed.

Another way is a cell density evaluation obtained by monitoring the consumption of oxygen or glucose (Kyung et al. 1994; Meuwly et al. 2006).

3.4.4 Cell bleeding

Cell bleeding is operated to partially remove the cells from the bioreactor, typically by pumping out the cell broth from the bioreactor. This operation is systematically included in a strategy where the cells are maintained at a stable cell density (see above). Three methods can be used:

- The more accurate method is to use a continuous pump automatically controlled based on the on-line cell density measurement in order to track the cell density set point.
- The continuous pump can also be manually tuned based on daily off-line cell density measurement achieving satisfactory results.
- Daily manual cell removal can also be used during the process development phase: the perfusion is momentarily stopped, cell broth is removed and then new fresh medium is added to compensate the removed culture volume before re-starting the perfusion.

Manual cell removals are also operated ad hoc to reduce the cell density, e.g. to respect given cell density limits of a registered process. During the process development, the studied conditions can result in degraded cell population with low viability and/or absence of growth; a manual cell removal is then applied to help the cell recovery. This can be accompanied (or not) by a momentarily increase of the perfusion rate in order to speed up the medium renewal, providing more favorable environmental conditions.

3.4.5 Cell arrest

Likewise in fed-batch processes cell arrest by physical or chemical means can be used, given that the cell specific productivity is increased and that the protein quality is correct - or even improved - (Angepat et al. 2005; Chotteau 2001; Oh et al. 2005). Cell arrest in G0/G1 phase can be reversibly obtained for instance by reducing the temperature (Angepat et al. 2005; Zhang et al. 2013) or adding a chemical like (toxic) butyrate (Oh et al. 2005), see Section 3.4, paragraph 'Stable cell density with arrested cells'. The (more abundant) knowledge reported for batch and fed-batch processes like the ranges of temperatures or butyrate concentrations can be applied in perfusion processes. These parameters have to be optimised on a cell line specific basis. Typically a lot of cell lines are still growing at 34°C or 35°C but slower than at 37°C and are barely growing at 31°C. As previously mentioned, these approaches can lead to increased cell damage.

3.4.6 Cell viability

Low cell viability can affect the POI quality due to the associated proteolytic activity released by the lysed cells. Another important effect of the presence of dead cells is the release of nucleic acid and cell debris, reported to play a major role in filter clogging (Esclade et al. 1991; Mercille et al. 1994). It is therefore highly advantageous to maintain the cell viability as high as possible. Finally, a consequence of dead cells is the accumulation in the culture of cell debris, which may be removed through bleeding or via some perfusion devices.

3.5 Protein quality

The POI present in the harvest is stored in a cooled harvest tank during the culture (unless continuous purification is employed), allowing a good preservation of the POI quality. For instance the proteolytic activity is highly reduced. During the process development, the evolution with time of the POI quality in the cooled tank is studied according to the quality attributes important for the POI, i.e. analyses/characterisation. This study together with logistics and COGS factors will contribute to the decision of the harvest frequency for the process. The constant environment of the perfusion greatly contributes to the stability of the quality attributes with time. Another factor of attribute profile variation with time is the application of cell arrest (see Section 3.4.5).

If small variations in the POI quality like minor variations in the distribution of species occur, a common procedure in industry is to pool different POI batches issued from different harvests from different culture runs in order to reconstitute the POI according to the specifications.

3.6 Parameter optimization

During the process development, the effect of the parameters on the process can be preliminary studied in batch mode or in pseudo-perfusion (see Section 2.1). This is then confirmed/further studied at bioreactor scale. During a bioreactor run, several parameter values can be tested sequentially in time (Miller et al. 2000; Hiller et al. 1993):

- a culture at a given constant cell density is established (see Section 3.4)

- the effect of a given parameter value is tested during several days, e.g. 4 to 7 days, by monitoring the cell growth, viability, metabolism, POI production and quality (if relevant)

- then this parameter is changed to a new value to be tested

- after a transition period, e.g. 2 to 3 days, the effect of this new value can be monitored as described above. Notice that in case the previous parameter value was extreme and damageable for the cells, the transition period has to be longer until the cell growth and viability are back to their normal values.

A factorial analysis, also called Design of Experiment, approach can advantageously be adopted to study the effect of several parameters on the process as commonly used in the whole culture process development field (Pinto et al. 2008; Bollin et al. 2011; Sandadi et al. 2006).

3.6.1 Environmental parameters

Different physical parameters have large or moderate influence on the process.

- pH: The pH has a major effect on the cell growth, POI, glucose/lactate metabolism and therefore alkali addition, osmolality and pCO_2 level. Often, different pH values are optimal for the cell growth and the POI production.

- Dissolved oxygen concentration (DO): The DO has often a much minor effect on the POI production given that DO is between some 20 and 80 % of air saturation (Link et al. 2004). Higher cell specific glutamine and glucose uptake rates have been reported for hybridoma with increasing DO (Jan et al. 1997; Thommes et al. 1993). Below 20 % DO, the cells are submitted to stress affecting the metabolic and production rates as well as the growth rate.

- partial pressure of carbon dioxide,(pCO_2): pCO_2 values in the range of 15 to 105 mmHg (2 to 14 kPa) have no major influence on the process. Larger values can affect the cell growth, the POI production and quality. An advantage of perfusion compared to fed-batch is lower values of pCO_2 provided by the medium renewal.

- Temperature: see Section 3.4.5.

- Scale-up factors, i.e. shear rate/shear stress, gassing-bubble damage: see 'Scaling-up and Tech transfer' Ozturk, SS

3.6.2 Substrate concentration optimization

It is recommended to maintain the glucose and glutamine at stable values during a production process to maintain the cell metabolism constant. Low levels of glucose, i.e. ≈ 5 mM, and glutamine, i.e. ≈ 0.5 mM, result in low production of lactate and ammonia so this is a valuable strategy for perfusion processes.

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Shaun Eckerle joined Centocor Biologics LLC in 2004 as part of cell culture manufacturing. Subsequently he moved to Manufacturing Technical Support for non-conformance resolution, technology transfer and manufacturing process improvement. Following the acquisition of the facility by Gallus BioPharmaceuticals, Shaun transitioned to Process Development leveraging his expertise with perfusion bioreactor systems to new processes developed at Gallus. Shaun has worked closely with Xcellerex to establish expertise of the Xcellerex disposable bioreactor technology to Gallus. Currently Shaun is developing processes to convert from traditional stirred tank bioreactor to Xcellerex Disposable technology, developing scalability from the XDR-10 to XDR-2000 as well as ATF System perfusion with the Xcellerex platform. Shaun has a B.S. Biological Sciences and is working on a M.S. Biochemistry.

05

Case Study: Optimized Perfusion

Abstract

Implementation of a perfusion process allows for improved and reproducible productivity within a low to mid-range producing system. Gallus optimized a bioreactor process for a Sp2/0 cell line producing a monoclonal antibody. The previously developed process was not providing optimal results. The understanding of metabolic need and proper perfusion optimization allowed for a 56% increase in cumulative bioreactor output.

Introduction

The perfusion bioreactor process is useful for generating high cell density cultures and when properly executed yielding improved bioreactor performance and cumulative output. In order to properly develop a perfusion process, understanding the metabolic needs of the culture is a requirement. Without proper understanding, the perfusion rates and bleed rates may not be operated to best maintain the metabolic need of the culture. Through understanding and adjustment, a perfusion process can be optimized and executed to maintain high cell density, high viability cultures with consistent and sustained antibody production. This case study demonstrates understanding the metabolic need and adjusting the perfusion parameters to better optimize a previously established process.

Materials and Methods

- Cell Line: Sp2/0 cell expressing monoclonal antibody
- Perfusion – ATF System
- Glass vessel operations: Applikon 5L / Sartorius DCU II
- Metabolite Analysis: NOVA BioProfile 400
- Cell Counting: Manual
- IgG Analysis: HPLC
- Glucose Feed
- Glutamine Feed

Procedure

- Inoculum scaled up in T-flasks , expanded to disposable cell bags
- Performance of 5L Glass Vessel with ATF2 to confirm process
- Optimization of Perfusion Process in 5L Glass Vessel with ATF2
- Scale up to 50L Stainless Steel with ATF6

Glucose and Glutamine Metabolism
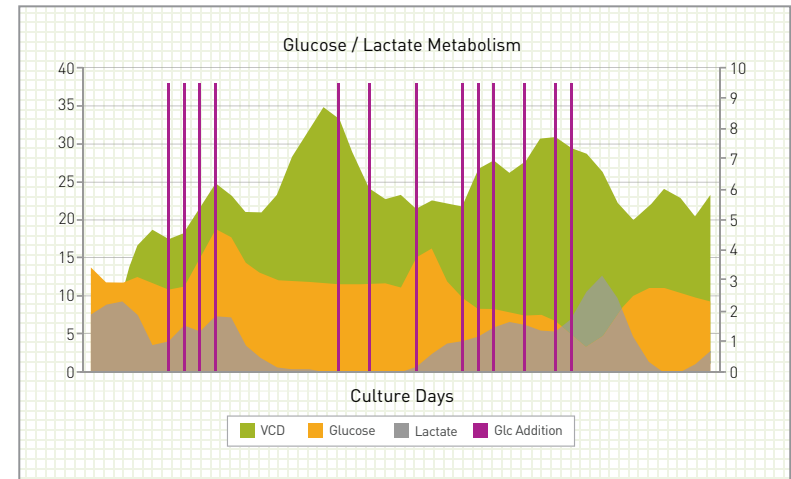
Fig. 1

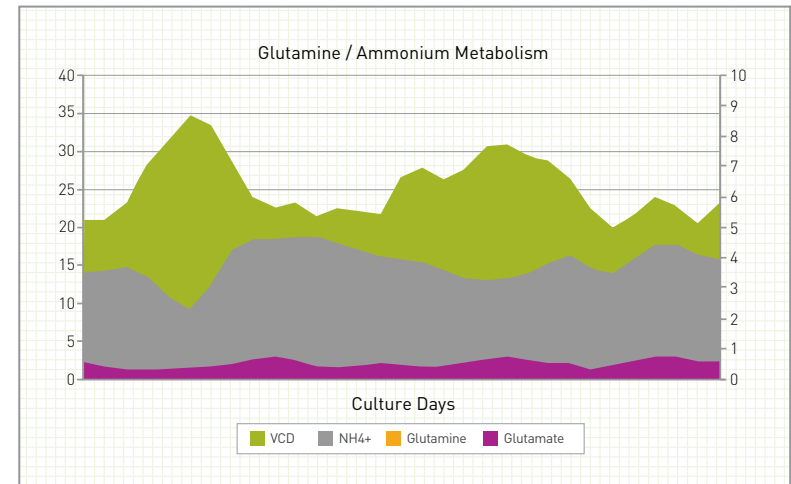- Inducing lactate consumption
  - Discontinue Glucose Feed

Fig. 2

- Inverse relationship between VCD and NH4+
  - Increase Gln in formulation, remove feed
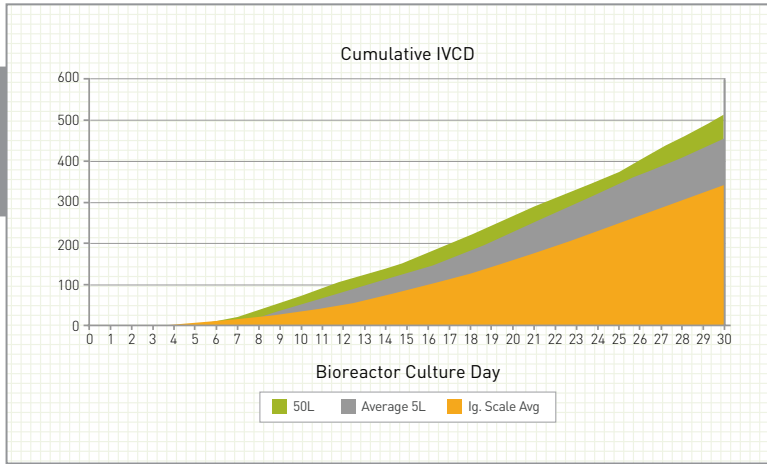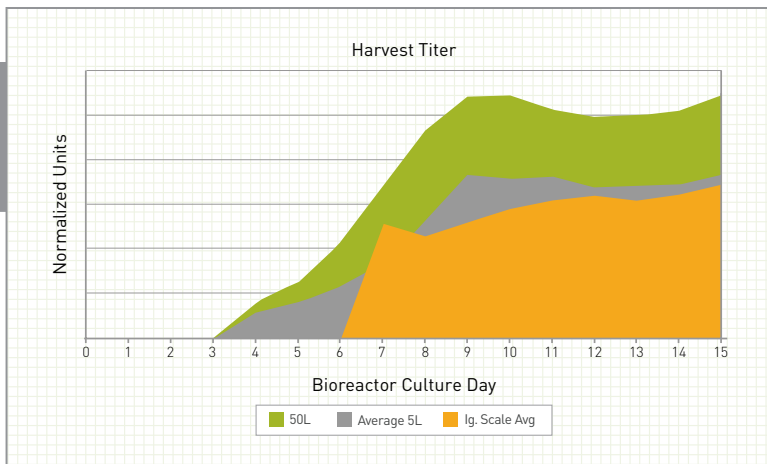
VCD and Antibody Improvements

Fig. 3

Cumulative IVCD

50L Average 5L Ig. Scale Avg


Fig. 4

Harvest Titer

50L Average 5L Ig. Scale Avg

Conclusions

By further understanding the metabolic need of the culture, the process was adapted to remove external feeds. The optimization of the perfusion process has lead to a more rapid increase in viable cell density and titer resulting in an average of 56% increase in cumulative bioreactor output at lab scale. Perfusion processes when optimized can lead to significant increases in product yield and ultimately reduction in cost of goods.

Fig. 5

Cumulative Harvest Output (5L)

Orig. 1
Orig. 2
Orig. 3
New 1
New 2
New 3
New 4
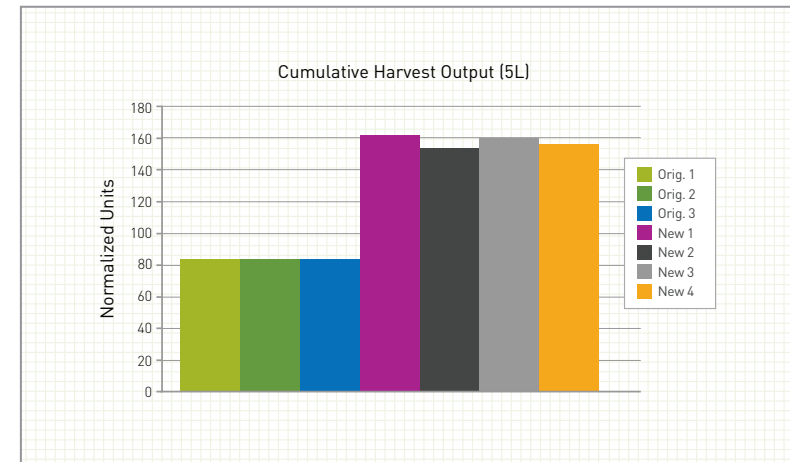
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Dr. de Jongh (South African) obtained a Bachelor degree followed by a M.Sc. in Chemical Engineering from the University of Stellenbosch, South Africa. Thereafter, he was awarded a doctorate in Biotechnology from the Technical University of Denmark in 2006. Dr. de Jongh has seven years' experience in the pharmaceutical industry in molecular biology; project management; process development; and process transfer to cGMP manufacturing.

06

Process Intensification Approaches for Cost Sensitive Protein Applications

The choice of a manufacturing platform and production mode are some of the most important strategic decisions in recombinant subunit vaccine development. *Drosophila* S2 insect cell expression is less known than the extensively used Spodoptera (Sf9) or Trichoplusia ni (Hi-5) insect cell based Baculovirus expression system (BEVS). Nevertheless S2 cells have been used in research for almost 40 years. The cell line was derived from late stage *Drosophila* melanogaster (Fruit fly) embryos by Dr. Schneider in the early 1970s, who named the cell line *Drosophila* Schneider line 2 (synonyms: S2, SL2, D.mel. 2). The S2 system has unique advantages for low-cost production compared to BEVS as it is a stable cell line, non-viral and a non-lytic system. This allows for a wide variety of upstream processing options compared to the obligatory batch process approach of the high-yielding, but lytic BEVS system.

The field of neglected diseases is specifically relevant for the application of process intensifying and cost reducing processing production modes. Particularly, the geographic distribution of malaria and the philanthropic funding sources involved require production to be as cost-effective as possible. Single-use bioreactors combined with perfusion production mode provide manufacturing flexibility and economic advantages, both highly desirable in this type of process. ExpreS²ion Biotechnologies aim to develop cost-effective *Drosophila* S2 based production processes combining its ExpreS² constitutive insect cell expression system with single-use bioreactor and perfusion technology.

ExpreS²ion has established collaborations with The Jenner institute, Oxford University and The Center for Medical Parasitology, Copenhagen University, to develop the protein production processes for the blood-stage malaria vaccine antigen protein (referred to as Protein2 in the text) and the placental malaria vaccine antigen VAR2CSA, respectively. The production of these complex protein vaccine antigens provides an ideal opportunity to apply advanced processing technologies.

Methods:

Batch, fed-batch and perfusion modes were compared for growth profiles and product yield. A truncation variant of the **VAR2CSA** placental malaria vaccine antigen and full-length **Protein2** were cloned into a pExpreS^2 vector and transfected into *Drosophila* S2 insect cells. Stable cell lines were established in three weeks using antibiotic selection in T-flask culture. The cells were expanded and inoculated at between 5E6 and 8E6 cells/ml for batch, fed-batch, or concentrated perfusion in 1L DasGip, 2L B Braun or CellReady3L bioreactors. The batch production runs were harvested after 3 days, fed-batch after 7 days and perfusion cultures after 6 or 9 days. An ATF System (alternating tangential flow) from Refine Technology was employed for concentrated perfusion production. The bioreactor conditions were 25°C, pH6.5, and 110-150rpm stirrer speed using a Marine impeller. The perfusion rates were set to 0.5 to 3 Reactor Volumes (RV) per day and was increased significantly faster for the CellReady 3L perfusion run compared to the B Braun runs, with 3 RV per day reached by day 6 vs. day 9 for the Braun runs.
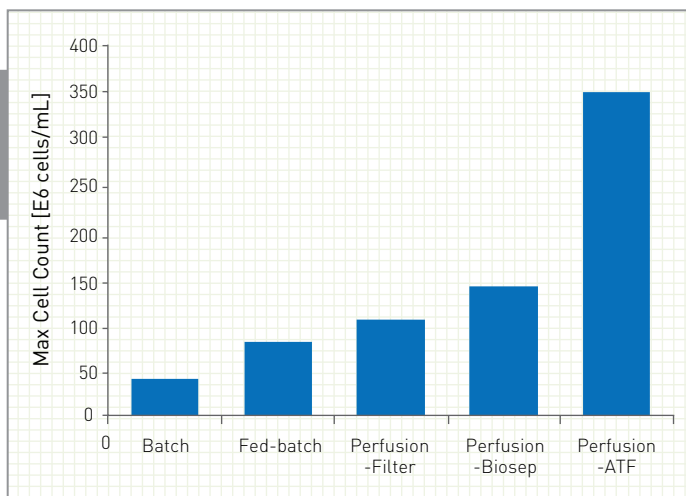
Fig. 1: Maximum cell counts achieved in different production modes.

The Perfusion-Biosep run was performed using the 10L BioSep (Applikon) in a 2L B Braun bioreactor and the Perfusion run was performed using the ATF2 (Refine) in a CellReady3L (Merck-Millipore). The Perfusion-Filter experiment (Wang et al. 2012) was performed using the Wave system (GE Healthcare) in a 2L CellBag using a floating filter with nominal pore size of 7 µm.

Results:

Cell counts achieved using perfusion technology

S2 cells normally grow to cell densities of 40–50E6 cells per mL in batch mode. A fed-batch approach can increase the cell counts to 60-80E6 cells per mL. However, further increases of up to 104E6 cells per mL have been reported (Wang et al. 2012) when using a floating filter in a wave bioreactor. ExpreS^{2}ion has achieved 140E6 cells per mL using the Biosep perfusion technology in 2L and 5L B Braun bioreactors. Recently, the application of ATF System perfusion technology has improved the cell density to 300-350E6 cells per mL in both B Braun 2L and Cellready3L bioreactors (Fig. 1).

Effect of feed strategy

Significant effects on growth and production were seen depending on feed strategy. The increased growth rate observed for the CellReady3L perfusion run compared to the B Braun bioreactor was due to a feed profile designed to allow maximum growth rate in the Cellready3L (see Fig. 2A). The feed profile for the B Braun run was designed to obtain linear growth. An exponential growth rate up to 350E6 cells/mL was achieved, and the production was only stopped because the maximum flow rate of the filter was reached. However, it is clear that the specific productivity of the S2 cells under exponential growth conditions was significantly lower when compared to the linear growth conditions. Similar yields were achieved on days 1 through 6 in both bioreactors, even though the cell counts were up to three fold higher in the exponential growth experiment (see Fig. 3). Similarly, a linear growth profile was maintained for the VAR2CSA concentrated perfusion run. However, on day 9 the perfusion rate was increased from 2 to 3 RV/day, which lead to a large increase in cell number. As this was the maximum possible perfusion rate with the ATF2, the increased cell count could not be maintained with an increased perfusion rate, which led to a drastic decrease in cell viability. This demonstrates the need to maintain a minimum perfusion rate to achieve high viability. ExpreS^{2}ion estimates the needed perfusion rate using the standard approach of attempting to maintain a constant flow rate per cell per day throughout the run.
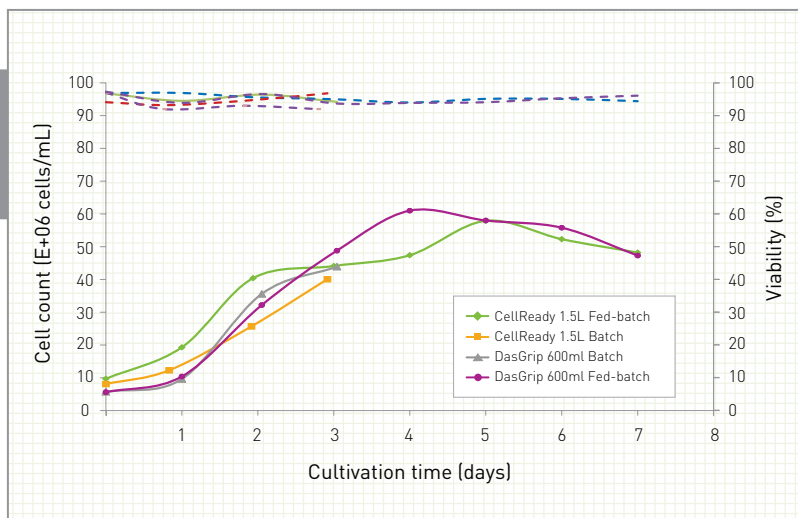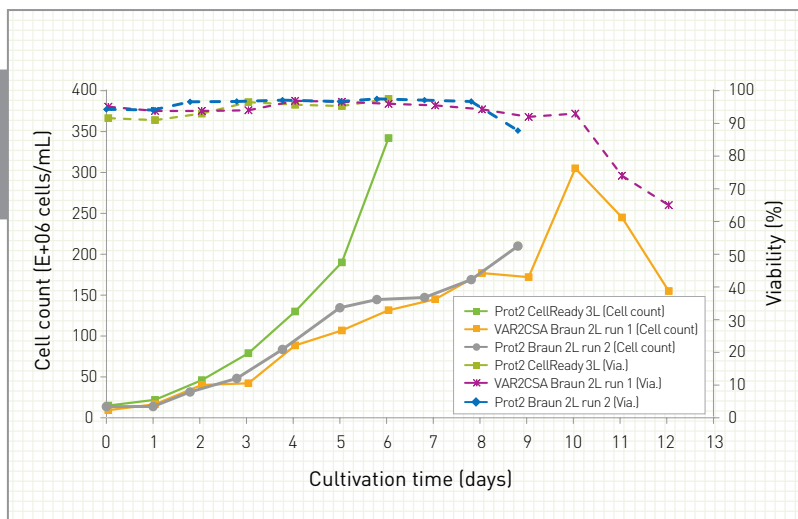
Fig. 2A

Fig. 2B

Fig. 2. (A) Growth curves for Batch and Fed-batch S2 cultures cultivated in either CellReady3L or 1L DasGIP bioreactors, (B) Growth curves for perfusion runs using the ATF2 in 2L B Braun or CellReady3L bioreactors for either a VAR2CSA or Protein2 producing cell line.

Yield improvements achieved using Fed-batch and Concentrated Perfusion

The VAR2CSA truncation variant was expressed in batch and fed-batch culture in 1L DasGip Bioreactors. A higher than 30% yield increase was achieved when using a fed-batch approach compared to batch production.

Concentrated perfusion was also performed on the cell line in a B Braun 2L bioreactor, and extremely high cell counts of 350E6 cells/mL were achieved. Unfortunately, no quantitative analysis technique was available to determine the yield increases achieved. An ELISA method is currently under development, but from western blot analysis it could be seen that significant yield increases were achieved.

The production of Protein2 was also compared in batch, fed-batch and concentrated perfusion using both CellReady3L and glass bioreactors. Significant yield increases were obtained going from batch to fed-batch production, and again from fed-batch to concentrated perfusion. Comparable yields were obtained in both CellReady3L and B Braun bioreactors (see Fig. 3). Furthermore, **350E6 cells/ ml** were achieved in concentrated perfusion mode using the ATF System and CellReady3L. Concentrated perfusion lead to final Protein2 yields of 210mg/L and **500mg/L** after 6 or 9 day production runs.
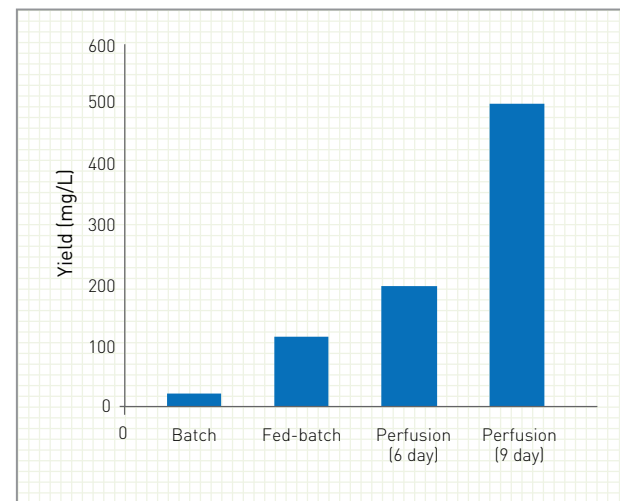

Fig. 3: Protein2 yield from Batch, Fed-batch and Concentrated perfusion cultures.

Protein stability:

Strikingly, it could be observed that decreased cell viability on the last two days of the VAR2CSA perfusion run due to a too low perfusion rate led to extensive product degradation. Clearly, this degradation could be avoided by maintaining cell viability. Similarly, SDS-page analysis of the purified of Protein2 from a day 8 harvest from Fed-batch culture, or a day 10 harvest from the perfusion culture, showed increased intensity of bands corresponding to two degradations product of Protein2 when compared to the fed-batch culture (see Fig. 4). In this latter case the product cleavage was less severe, although it was also present even while the culture was maintained at high viability.

Fig. 4
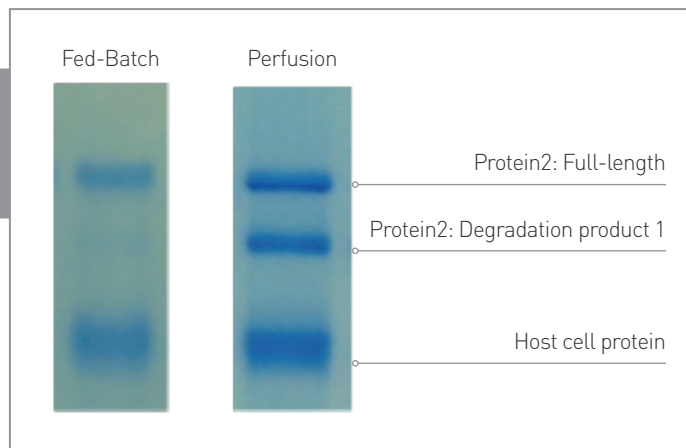
SDS-page analysis of purified Protein2 from a day 8 harvest from Fed-batch culture, or a day 10 harvest from the perfusion culture.

Conclusion:

The protein stability issues observed for Protein2 (and to a lesser extent for VAR2CSA) demonstrate one of the key weaknesses of concentrated perfusion technology, namely the need for product stability to enable extended product residence times in the bioreactor. However, for degradation prone proteins, the option of performing standard perfusion using the ATF System offers a simple solution by reducing the product residence time to less than 24 hours with direct harvest at 4°C. It is therefore necessary to evaluate each protein based on

stability before deciding on applying either concentrated perfusion or standard perfusion. For instance, the VAR2CSA truncation variant could be successfully produced using concentrated perfusion on the condition of high cell viability. Significant yield increases through increased cell counts, and consequent production scale reductions are possible in both cases. Concentrated perfusion or standard perfusion using the ATF System therefore offers attractive process intensification approaches for cost sensitive protein production needs.

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Bill Whitford is Sr. Manager, Emerging Technologies for Thermo Scientific Cell Culture and Bioprocessing in Logan, UT with over 20 years experience in biotechnology product and process development. He joined the company nine years ago as a team leader in R&D developing products supporting biomass expansion, protein expression, and virus secretion in mammalian and invertebrate cell lines. Products he has commercialized include defined and animal product-free hybridoma media, fed-batch supplements, and aqueous lipid dispersions. He had previously served as Manufacturing Manager for PHASE-1 Molecular Toxicology in Santa Fe, New Mexico producing custom and standard DNA microarrays. An invited lecturer at international conferences, Bill has published over 200 articles, book chapters and patents in a number of fields in the bioproduction arena. He now enjoys serving on the Editorial Advisory Board for BioProcess International

07

Impact of Single-use Technology on Continuous Biorocessing

Single-use in Bioprocessing

Single-use in bioprocessing refers to materials or equipment that can be used in one processing batch or campaign, and usually having a product contact surface element that is disposable. Such equipment ranges from single material, very simple stand-alone items such as a tubing– to complex and controlled systems of many components and materials, such as a bioreactor [1]. Relatedly, the application of such equipment ranges from an instrument with a single, simple function to skids housing entire or even combined unit operations. Most of the more complicated single-use (SU) systems contain re-usable non-product-contact elements, for such purposes as support. SU systems have been taken up in the biopharmaceutical industry in general because of the numerous features they provide (Table 1A). Over the past 10 years or so the number of individual process activities, upstream operations support – as well as entire systems available has grown substantially (Table 1B). Some of the newer products available for upstream applications include disposable pumps, single-use flowpath auto-sampling and microcarrier separators [2].

Table 1. Upstream single-use technology features and systems

A: Features Provided by SU	**B**: Operations Supported by SU
Reduced contamination risks	Cell culture for seed expansion and production
Lower initial investment costs	Media , buffer and process liquid preparation
Lower facility and operating cost	Liquid pumping, filtration, collection, shipping
Reduced operator requirement	On-line contents monitoring sensors/samplers
Process efficiency and flexibility	Transport/storage of intermediate and product
Time to market and ease of use	Cryopreservation of seeds and intermediates

Continuous Processes in Upstream Bioproduction

By far the most common approach to continuous processing in upstream animal cell-based bioproduction is through perfusion culture [3, 4]. In perfusion culture medium is added at rates exceeding the cell mass expansion rate and the excess medium is removed using some device to retain cells in the bioreactor [5, 6]. A number of such research- and production-scale perfusion bioreactor systems have been devised [7]. Although many perfusion processes for either suspended or adherent Animal cells are known to be used in manufacturing-scale production, details on their design and operation are not always publically available. Terminology in this dynamic field can get fuzzy, for example, continuous processing is also referred to as continuous production, continuous flow processing or continuous manufacturing. Minor distinctions are sometimes made with them. Depending on the periodicity of either entire production episodes or of more discrete individual component operations, some even apply such terms as semi-continuous or pseudo-continuous operation[8]. Nevertheless, interest in the field is growing [9-12] significant stakeholder investment is occurring [13] and commercial instrumentation to support its incorporation in single-use or hybrid applications is now appearing.

Single-use in Continuous Bioproduction

SU technologies supply a number of values to any mode of bioprocessing, but can provide some specific and enabling features in continuous bioprocessing (CB) implementations [14-17]. CB has introduced an interesting twist on the standard paradigm of the concept of iterations of equipment usage. There has always been a bit of wiggle in the distinction between the concept of "single-use" and such terms as "disposable" or "limited-use". Presented here is an introduction of how CB has determined a re-examination of a few related concepts in this regard (Table 2) and how SU and hybrid equipment supports such upstream CB approaches as intensified perfusion culture.

Table 2. CB-modified qualifiers of usage in biotechnology

Concept	Definition	CB-specific Modification
Reusable	Equipment or material intended for use in a process for an indefinite number of times: especially in different production cycles or batches, and after salvaging or preparation by special treatment or processing.	None
Multi- or limited-use	Equipment or material intended for use in a process for a limited number of times: determined by validated procedure or subsequent testing.	As CB by definition can increase the time and throughput volumes involved in each "use", review of the number of iterations addressed is advised.
Single-use	Equipment or material intended for use in a process for one time and then retired from use.	As CB by definition can increase the time and throughput volumes involved in each "use", review of the validation requirement is advised.
Hybrid	Equipment or material composed of both reusable and single-use components.	None
Disposable	Equipment or material intended for use in a process either for one time or for use in a process in a limited number of times, and then retired as waste or garbage.	Same alteration as either "Single-use" or "Multi-use", depending upon the intent.

Most every operation in a CB process train is now supported by a commercially available single-use, or at least hybrid, solution. First of all, many of the SU equipment and solutions being developed for batch bioproduction have the same or related application in CB systems. Examples here include simple equipment such as tubings and connectors, to more complex applications such

as the cryopreservation of large working stock aliquots in flexible bioprocess containers (BPCs). The list of CB-supporting SU technologies being developed is large and growing (Table 3)

Table 3. Continuous Bioproduction Related Single-use Technologies

Preparation and storage of media/buffers in SU mixers
SU liquid and gas filtration of many types, including TFF
Storage of media and buffers for CP feeding in SU BPCs
Distribution of process fluids in metered SU manifolds
SU storage and metered distribution of dry powders
SU or hybrid bioreactor cell culture in seed generation
Production in SU or hybrid-SU perfusion bioreactors
Continual appearance of new SU probes and sensors
SU real-time automated online multi-analysis interface
SU flow-path on-line real-time controlled feed porting
Bulk harvest by SU centrifugation or filtration into BPC
Purification in SU traditional or PCC chromatography
Final fill in SU and/or automated and closed apparatus

A SU advantage in process development is its supports of an open architecture approach and a number of hybrid designs. Such designs include combining reusable and single–use systems, or between divergent suppliers of particular equipment. Especially in bioproduction, the many flexibilities of SU support a manufacturing platform of exceptional efficiency, adaptability, and operational ease [18]. Advanced solutions in SU transfer tubing, manifold design and container porting also supports creativity in process design. This is of particular value in designing a process with such demands as entirely new flow paths or lot designations, such for CB.

SU systems upstream provide a reduced footprint and eliminate of the need for cleaning and sterilization service. This complements perfusion culture's inherently smaller size and independence from cleaning for extended periods of time.

Several newer approaches to formulating process fluids support the concept of CB. Single-use mixing systems are typically constructed of a rigid containment system with a motor and controls driving radiation-sterilized single-use bags equipped with disposable impeller assemblies. From a variety of manufacturers there are a number of distinct approaches to motor/disposable impeller assembly linkages, tubing lines and connections. Also appearing are a number of exciting SU sampling, sensing, and monitoring solutions. Single-use powder containers permit seamless transfer between powder and liquid formulation steps, and the ridged mixing containers are available in jacketed stainless steel for heating and cooling requirements. Surprisingly, the "topping-up" of large-scale single-use fluid containers with newly prepared buffer to provide a virtually unlimited and constant supply of each buffer/media type can be validated for GMP manufacturing procedures.

Continuous, automated in-line culture media and buffer dilution and conditioning have been attempted for decades, and interest in them remains high [19]. Of late, advancements in the in the mass flow technology, monitoring and feedback control required to establish and maintain process fluid specifications are now allowing such approaches to become a reality [20]. The compact size and portability of the equipment involved allows it to produce fluids at the "point of use" and is supported the incorporation of SU. So, in-line preparation and fluid conditioning provides benefits to bioprocessing in general, supports CB in particular and contributes specific features supporting single-use technology application in CB. For example, its demand for significantly reduced buffer prep tank sizes supports application of single-use BPCs containers and manifolds.

Process flexibility is a key feature in both SU and CB. CB contributes to overall process flexibility in that equipment tends to be easy to clean, inspect and maintain – and generally promotes simple and rapid product changeover. SU systems can provide similar flexibility and ease product changeover because

they tend to be more modular and transportable than much of the older batch equipment. In fact the size, configuration and reduced service requirements of SU systems actually encourage diversity of physical location within a suite or plant, as well as re-location to other manufacturing sites.
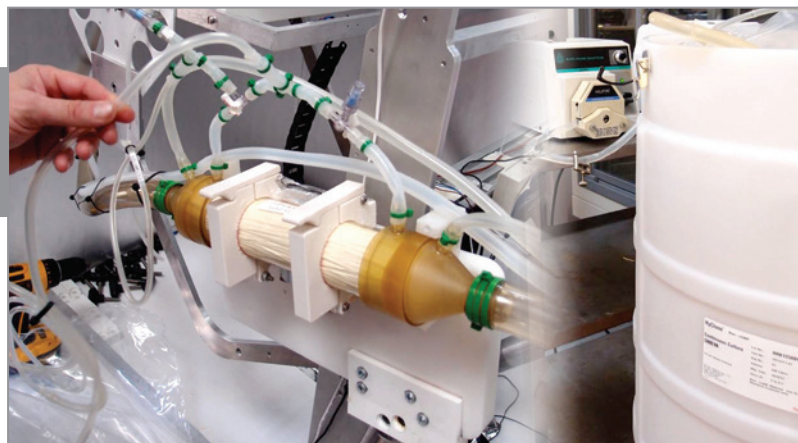
Fig. 1

Single-use intensified perfusion-based continuous bioproduction using a single-use Thermo Scientific HyClone bioprocess container and drum (right) supporting large-scale culture in a FiberCell Systems LS-HFBR hollow fiber perfusion bioreactor (left)

Due to its inherent demand for immediate process data and control capabilities, CB supports initiatives in continuous quality verification (CQV), continuous process verification (CPV), and real-time release (RTR) [21-23]. Although CB will not be feasible for all products and processes, many implementations well-support a "platform" approach, in which a single process supports more than one product. CB most always shortens the process stream, reduces downtime, and greatly reduces handling of intermediates. These features synergise with the operational efficiencies of SU systems, contributing to a greatly reduced cumulative processing time for the API. Furthermore, they greatly simplify production trains and inherently facilitate application of closed and integrated processing approaches to individual operations and even processes. Especially in bioproduction, the modularity and integral gamma irradiation sterility of SU combined with the sustained operation of CB promise the appearance of platforms of unparalleled operational simplicity and convenience.

The heart of a CB approach is the bioreactor. Perfusion bioreactors have been successfully employed in bioproduction, even biopharmaceutical production, for decades. And, rather remarkably, disposable bioreactors have been available for nearly 20 years. At the research scale there have even been single-use hollow fiber perfusion bioreactors available from a variety of vendors for over 40 years. However, only recently have commercially available SU and hybrid production-scale perfusion-capable equipment become available [24-26].

Fig. 2

Hybrid continuous bioproduction accomplished in a Thermo Scientific HyPerforma S.U.B. TK 250L (left) supported by a Refine Technology ATF System (right).

The production-scale CB enabling SU bioreactor technologies now appearing include single-use and hybrid perfusion-capable reactors (Fig. 1, 2); a growing variety of SU and hybrid monitoring probes and sensors; SU pumps and fluid delivery automation of various design; and automated SU online sampling, interface, valving and feeding technologies. Their coordinated implementation in actual production settings with appropriate control is now beginning.

Justified or not, concerns in the implementation of CB include performance reliability (incidence of failure), validation complexity, process control and economic justification [27-29]. But for many processes, such previous limitations — or their perception — are being alleviated by advances in CB processing

technology and OpEx driven advances bioprocess understanding, reactor monitoring and feedback control [30, 31]. However, while some CB attributes inherently provide immediate advantages (such as reducing reactor residency time) others do present challenges (such as cell-line stability concerns).

Due to the limited contribution of API manufacturing to small-molecule pharmaceutical cost, the limited bottom-line financial savings of CB has been a concern. However, biopharma is a different animal in general, and as such trends as globalization and biosimilars alter the picture even further, the financial benefits of CB are becoming even stronger [32].

The fact that many SU systems are constructed of standards compliant and animal product-free materials supports CB applications in a wide variety of product types and classification. In fact, SU systems are available for most process formats (eg, microcarriers and suspension), platforms (eg, cell line, vectors, culture media), modes (eg, dialysis or enhanced perfusion) or scale (eg, through rapid, inexpensive scale-out). "Futureproofing", or supporting the sustainability of a new CB process in the face of product lifecycle or emerging technology imperative, is supported by many SU features [33]. Examples here include SUs low initial facility, service and equipment cost and especially undedicated manufacturing suits and ease of process train reconfiguration.

As advanced processing solutions are applied to single-use perfusion mode-capable reactors, the design of closed, disposable, integrated and continuous upstream bioproduction systems are finally being realized.

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08

Continuous Multicolumn Chromatography Processes

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Marc Bisschops is the Chief Scientific Officer of Tarpon Biosystems. Tarpon Biosystems is a biotech start-up company based in Boston that develops and commercializes a fully disposable continuous multicolumn chromatography technology.

Marc has 15 years of experience in the (bio-) pharmaceutical industry. He has been involved in process development and process improvement projects throughout most of his career. He held scientific and management positions within contract service organizations that focus on process development and process improvement projects for (bio-) pharmaceutical products. He is one of the world experts in the field of continuous chromatography and simulated moving bed (SMB) technologies and has been involved in more than 150 projects in this field worldwide. Marc is (co-) inventor on many patents related to process improvements and/or innovative biomanufacturing technologies and is an invited guest speaker at various post-graduate courses.

Before joining Tarpon Biosystems, Marc has headed biopharmaceutical process development and process improvement departments in Batavia Bioservices, Xendo and Univalid. Marc holds an MSc in Chemical Engineering and a PhD in Biochemical Engineering from Delft University of Technology (NL).

Introduction

Chromatography has been, and will probably remain for the foreseeable future, the most important workhorse in the purification of biopharmaceutical products. A wide variety of chromatography products are commercially available, offering the possibility to separate the product of interest based on affinity interactions, electrostatic interactions (ion exchange), hydrophobic interactions, size and combinations of these. In the biopharmaceutical landscape, there is not a single product that is not purified using at least one chromatographic purification step and most biopharmaceutical products require at least two chromatographic purification steps.

Although powerful in terms of removing contaminants, chromatographic processes have a few disadvantages. In general, multi-step batch processes exhibit poor productivity and often this leads to scalability limitations. Even capture processes with new high capacity chromatographic media cannot always cope with the high titers that are becoming more prevalent in cell culture processes. For example, a fed batch cell culture bioreactor of 2000 liter with a 5 gm/L expression level produces more antibody than can be bound on a column with a one meter diameter, even if that column is cycled twice per batch.[1]

In other process industries, these limitations have been successfully dealt with by implementing continuous multicolumn chromatography processes. Notable examples of this approach is the use of simulated moving bed (SMB) technology for separating fructose from glucose and many chiral separations common in purifying API's made through organic synthesis. Although the traditional simulated moving bed technology is mainly applied for binary fractionations, continuous multicolumn chromatography systems have also found large scale applications in capture processes. Examples of these are the purification of L-lysine and antibiotics from fermentation broth and the production of ascorbic acid (vitamin C).

With increasing cell culture expression levels, the capacity bottleneck in biomanufacturing has shifted from the upstream process to the downstream process. This has generated a need to address the limitations of batch chromatography in biopharmaceutical applications. This has resulted in various designs for multicolumn chromatography systems for the purification of biotherapeutics.

Key Features

The principle of multicolumn chromatography is to create a (simulated) movement of the chromatography columns in opposite direction of the process solutions. This results in a countercurrent contact between the liquid and the chromatography media, which allows overloading the columns beyond the dynamic binding capacity without suffering loss of material. When product breaks through from the first column, it will be captured on a second column in the load zone. With this, countercurrent chromatography processes can offer a significant gain in capacity utilization.

Another benefit of the countercurrent contact approach is that it eliminates idle zones in the process. In a batch chromatography column, the mass transfer zone only covers a small portion of the overall chromatography volume. The media above the mass transfer zone is in equilibrium with the feed solution and has no additional capacity to bind more product. The media below the mass transfer zone is in contact with depleted feed solution and hence is waiting for the first product to arrive. In a countercurrent process, these idle zones are eliminated and the load zone can be designed to only cover the length of the mass transfer zone. This is generally corresponds to a small part of the batch column volume. These two features are schematically demonstrated in figure 1.

In batch processes, the column size is proportional to the total mass of protein that needs to be purified and hence there is a direct relationship with the feed concentration. In a continuous chromatography process, the load zone is mainly designed around the contact time associated with mass transfer zone. The total volume of chromatography media in the load zone thus hardly depends on the static binding capacity and the feed concentration. Instead, the process is designed around the volume that needs to be processed, or more precisely, the feed flow rate.
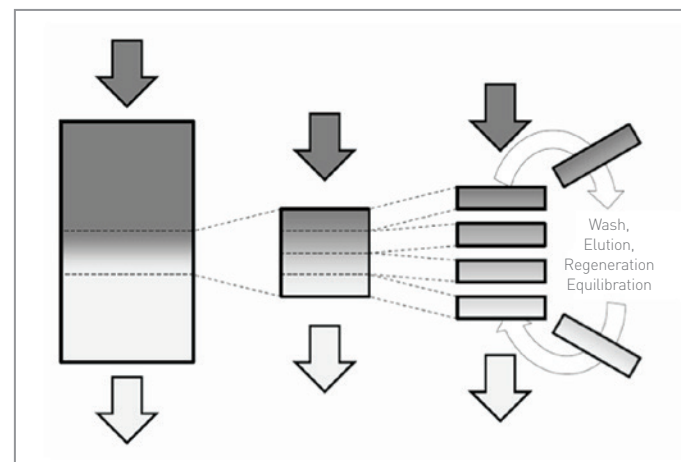

Fig. 1

Schematic comparison between a batch process (left) and a multicolumn countercurrent chromatography process (right).

The number of columns that is required to run a continuous process does depend on titer. In order to transform the load step into a continuous countercurrent step, at least two columns are needed. This brings the minimum number of columns for a continuous process to three, provided that one column provides sufficient time to do all wash steps, elution, regeneration and re-equilibration steps. As soon as the load volume becomes relatively low, which is the case for medium and higher titers, the load time becomes proportionally smaller and one column is no longer sufficient. For these scenarios, the ability to connect extra columns to the system without adding complexity to the valve system is a valuable attribute. For polishing processes, where the chromatographic resolution is not as straightforward as in affinity separations, additional columns may also be needed in the elution zone and/or wash zones.

Continuous and disposable

There is a substantial gain in specific productivity over batch offered by continuous chromatography while the size of the overall chromatography system – including its columns – becomes significantly more compact. The columns are cycled many times throughout each batch, usually up to the life time of the chromatographic media. This process design enables a viable disposable chromatography process.

The BioSMB® technology developed by Tarpon Biosystems is designed around a completely disposable product contact/ fluid path. Most importantly, the Tarpon BioSMB® valve cassette, a single-use acrylic block containing all the valving and integrated fluid connections to run a multi-column process, can operate up to 16 columns or other single use devices such as membranes or monoliths. Each of the valves in the cassette can be individually addressed, thereby providing all the flexibility that is required to operate virtually any chromatography process in a multicolumn configuration. In addition to this, the pumps, tubing and sensors are also available in disposable format.

When the BioSMB system is combined with prepacked columns, membrane adsorbers or any other chromatographic devices designed for single-use applications, the entire chromatography process can be translated into a viable single-use option. With this, the BioSMB® technology provides a promising answer for those companies who are developing completely disposable strategies for the entire biomanufacturing process.

Fig. 2
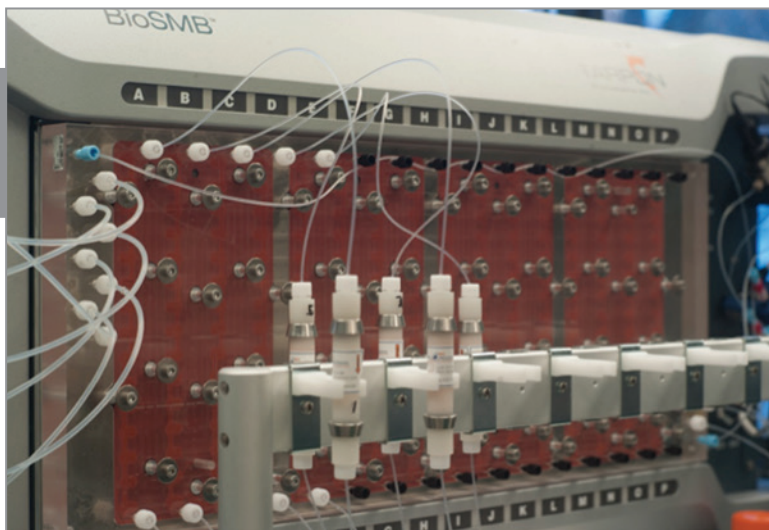
A BioSMB system for process development use with five prepacked chromatography columns connected to the system.

Optimization Strategies

Continuous chromatography processes have more degrees of freedom than batch chromatography. This offers more flexibility in optimizing the process to meet the specific requirements of each manufacturing situation. For instance, in continuous processing, the batch processing time becomes a choice rather than an outcome of the design procedure.

Clinical Manufacturing

In clinical manufacturing, the cost contribution of the consumables such as the chromatography media to the total COGs (Cost of Goods) is quite significant. This is mainly due to the fact that these consumables cannot be exploited to their full extent. Even expensive chromatography media such as Protein A affinity media are depreciated within a single clinical manufacturing campaign. The optimization strategy for this situation should target the total installed volume of chromatography media or the specific productivity (expressed as grams of protein purified per liter of chromatography media per hour).

In a BioSMB process, this translates into process conditions that target a short contact time between the liquid and the chromatography media. This can be achieved by operating below the highest possible capacity utilization and in the process accepting sub-optimal savings in buffer consumption. In clinical manufacturing, the buffer consumption is generally not the limiting factor and the impact of the total costs of the campaign is negligible in most cases.

Commercial Manufacturing

In commercial manufacturing, chromatographic media is depreciated over many more cycles than in clinical manufacturing. It is not uncommon to validate media life time up to 100 or even 200 cycles. In these situations, the cost contribution of the chromatography media is no longer related to the specific productivity of the process, but to the amount of product that is purified in each cycle per liter of chromatography media. This optimization strategy for continuous multi-column chromatography thus targets capacity utilization optimization. With this, the savings in buffer consumption will also be optimized.

In order to achieve the maximum capacity utilization, the load step will require a certain contact time, which has an impact on the specific productivity. In commercial manufacturing, however, the specific productivity does not affect the COG other than through capital costs.

Fig. 3
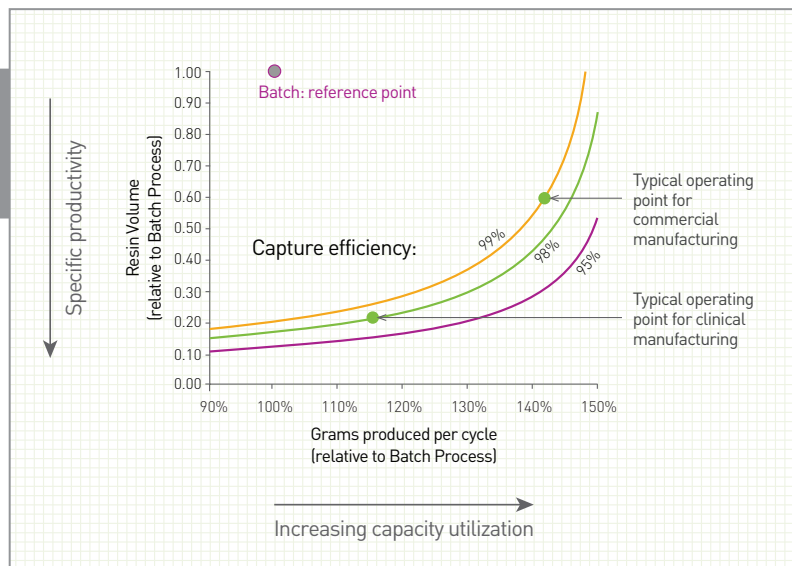
Schematic representation of the typical operating points for clinical manufacturing and commercial manufacturing in a Protein A based BioSMB process.

Application Areas

The flexibility offered by the BioSMB disposable valve system makes it a very versatile technology, allowing a wide range of applications. For various chromatographic processes, the impact of the technology has been investigated. A brief summary of some of the case studies is listed in Table 1.

Case study	Chromatographic mode	Specific productivity
Capture of antibodies	Protein A chromatography	2 – 6 x batch
Aggregate removal	Hydrophobic interaction (HIC)	2 – 3 x batch
Aggregate removal	Ion Exchange	4 – 8 x batch
Capture of recombinant proteins	Ion Exchange	2 – 5 x batch
Capture of VLP vaccines	Ion Exchange	3 – 7 x batch
Polishing of a VLP vaccines	Size Exclusion (SEC)	6 – 14 x batch

Table 1: Some examples for which BioSMB technology has been successfully tested [2, 3, 4, 5, 6].

The case studies listed above were performed with traditional chromatography media in prepacked columns. In addition to this, the combination of BioSMB technology with alternative chromatography formats has been successfully demonstrated. This includes the use of monolithic columns and membrane adsorbers to establish a continuous capture process. This has been done with membrane adsorbers ion exchange and with affinity ligands.

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Andrew has over 30 years design and operational experience in the biopharmaceutical industry. He founded Biopharm Services in 1999 to develop a technology-based services business focused on all aspects of bioprocessing. Since then he has helped a wide range of clients to better understand the value of new technology to their manufacturing bioprocesses.

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Andrew has significant experience in the simulation and economic modelling of biochemical and chemical processes and has worked with a wide range of client to assess the economic impact upon bioprocesses of new technologies, process definitions and manufacturing strategies. Prior to joining Biopharm Services, he attained an Engineering Doctorate in Biochemical Engineering from University College London.

09

Continuous Processes
Economic Evaluation

Introduction to cost modelling

Process models are tools to analyze processes and manufacturing options and support decision making. They have been used in our industry to:

- assess the cost of outsourcing;
- evaluate and screen process development options;
- help develop capacity and expansion strategies;
- compare existing and novel processing/manufacturing technologies

In this section we examine the use of cost modelling in continuous bioprocesses, focusing on the approaches adopted for the evaluation of batch and continuous operations. Most cost models draw on the principles of financial and management accounting to assess the cost impact of different investment and operating decisions. For manufacturing, the most significant line item on the income statement is the cost of producing goods for sale (referred to as Cost of Goods Sold or Cost of Sales) which is shown directly below net sales revenue. Subtracting the cost of goods sold from the sales revenue gives a company's gross profit, making it possible to evaluate manufacturing performance as a distinct measure that contributes to overall business performance. This is important from an executive management perspective because improvements in manufacturing performance that result in increased gross margin are made visible. A robust, well-structured cost model enables managers to have a better insight into the key cost drivers of the manufacturing process as well as the sensitivity of overall cost of goods to changes in these key parameters.

Cost of Goods (CoG) is by far the most commonly used method. While it has the merit of being the most familiar to people in the industry, it is not the most rigorous. CoG should not be used where there is a need to understand the interplay between the expenditures and project risk. Net Present Value (NPV) methodology is the best technique to analyze alternative technologies and manufacturing strategies, as it can account for the impact of delays in expenditures and properly account for the time value of money.

The industry has traditionally employed batch processing to manufacture the biological drug substance and has sometime used continuous perfusion operation for the production bioreactor. In perfusion fresh media is continuously supplied to the bioreactor whilst drawing off the cell free content from the bioreactor at the same rate. Historically, the decision to go this route has been linked to the product: where the protein is inherently unstable, perfusion operation is the preferred methodology. Perfusion adoption is largely driven by customary practice rather than evidence. Although there has been discussion on whether perfusion or fed batch is the more productive, there has been little published quantitative cost analysis to support either option for commercial production.

We are now at a stage where there is strong interest in the use of continuous technologies for bioprocessing, based on perceived advantages relating to

- Cost
- Smaller facility footprints
- Flexibility
- Better process control/product quality

In this section we investigate the comparative cost of goods and the capital requirement of continuous process versus batch processing. This will be analysed using the BioSolve Process (BSP) cost modelling package. BSP is used as it has the capability of modelling both continuous flow and batch operations in the same framework, allowing easy comparison of the operating modes.

BSP generates cost of goods estimates through the scaling and costing of resources from a process description that includes recipe components and scaling rules. This is illustrated in figure 1. The operation modes dictate how the model deals with equipment and resource allocations
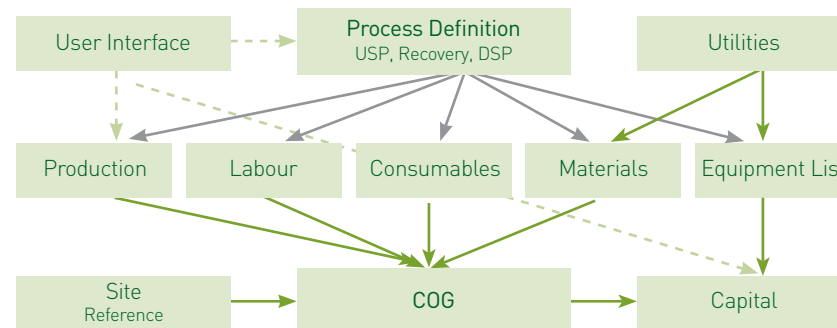

Fig. 1: Structure of a cost of goods model

Batch processing. The downstream processes sizing is based upon the pooled harvested product processed within a defined period. The batch scale is determined by bioreactor volume, bioreactor numbers, product titre and the bottleneck batch cycle time (for cell culture this is the bioreactor). This means that each operation is required to process the batch in a set time dictated by the harvest bottleneck. Only a small proportion of the batch time is used to process product: the remainder is associated with activities such as preparation, cleaning and regeneration.

Fully continuous processing. When steady state production has been achieved, the process will be continuously fed with a product flow, scale is determined by rate and product titre. The rate of product generation is the basis for sizing the unit operations and the resources required. Typically we are running operations with a finite capacity in a continuous line (filters, chromatography resins) membrane absorbers, TFF, etc.). The lifetime is determined by the capacity of the consumable. The unit operation is designed so that it can always receive flow. This can be achieved two ways.

- Switch over to a fresh device (two devices running in parallel)
- Put in surge capacity whilst a fresh device is being installed

Hybrid processing. At some point in the continuous line, a product may be collected as a batch. This could be after the capture column or at end of the process prior to formulation. There is a requirement to switch from continuous

mode to batch mode. BSP allows the user to determine this point by use of a switch operation, allowing the user to specify the amount material that forms the batch for subsequent processing. In addition BSP provides a switch that allows upstream batch operations to be converted into a flow for processing in a continuous line. This approach allows the model user to evaluate many different operating scenarios, for example

- Run perfusion and capture continuously and run the rest of purification as batch operation
- Have batch bioreactors feed a continuous DSP operation
- Run a continuous bioreactor and downstream, batching prior to formulation
- Run one unit operation continuously in batch process (chromatography for example)

BSP allows the user the flexibility required to fully evaluate continuous and batch operations from an economic perspective.

Configuration	Bioreactor		Rate VVD	Titre g/L	DSP Recovery
	500 kg/yr	2000 kg/yr			
Batch	4 x 1600	4 x 6500	N/A	4.5	60%
Hybrid	4 x 1600	4 x 6500	N/A	4.5	61%
Continuous	2 x 875	2 x 3750	2	0.9	74%

Fig. 2: Scenario definition for batch, hybrid and continuous process

Modelling Batch vs. Continuous

The biopharmaceutical industry was developed around the concept of batch processing. Though some companies have experience of operating perfusion bioreactors, there is little experience in the operation of downstream continuously. The development of fully continuous processes, with a perfusion bioreactor downstream running in parallel has become of interest, and we see many companies beginning to establish these set-ups at a pilot scale to investigate the feasibility of running in this operational mode.

Modelling packages such as BSP are based on commercial scale cost data sets and are widely used to evaluate process and technology choices in the industry. This makes them an ideal tool for evaluating new innovative technologies from an economic perspective, providing valuable insight into the impact of a new technology and how best to maximise its value. To illustrate this we have used the BSP package to estimate cost of goods to manufacture a monoclonal antibody bulk drug substance for a range of scenarios to better understand the impact of continuous operation on MAb production costs. The manufacturing scenarios considered are summarised in figure 2. The objectives of this study are to understand the impact of scale on the economics, to evaluate the relative contributions of upstream and downstream processing and to gain insight into the impact of continuous processing on the cost structure. Three process configurations were considered figure 3:

- Batch with fed batch upstream and batch downstream
- Hybrid with fed batch upstream and continuous downstream
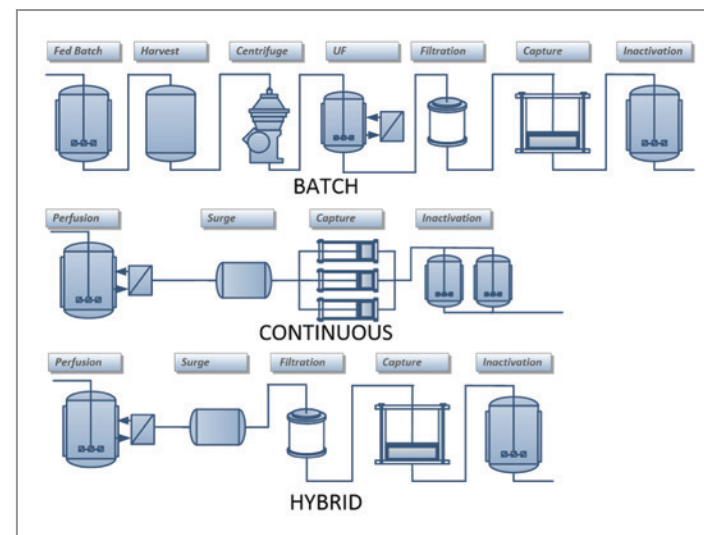- Continuous with perfusion upstream and continuous downstream

Fig. 3

Scenario schematic flow

Two manufacturing scales were considered for each process scenario based upon an annual requirement to manufacture 500kg or 2000kg. Multiple bioreactors were used for each scenario with the scale of equipment varying with annual throughput as illustrated in figure 2. Each bioreactor is harvested in parallel to feed the respective downstream line. The titre from the fed batch bioreactor was assumed to be 4.5g/L in line with the capabilities of modern cell lines. The perfusion culture was assumed to generate 0.9g/L at a perfusate rate of 2 vessel volumes per day.

An overview of the BioSolve Process estimate of the manufacturing cost structure at a scale of 500kg/yr. is given in figure 4. The cost is broken into the key cost categories that make up the overall cost of goods:

- Capital
- Materials
- Consumables
- Labour
- Other (waste, maintenance etc.)

The first interesting observation is that the upfront capital investment is significantly reduced by about 60% for continuous operation at both scales (Fig. 4). The reduction is seen in both upstream and downstream: on close examination we find that this is driven by the much smaller scale of operation required for the continuous facility. According to the model, the peak flow onto the batch protein A column is about 2800L/hr.; this reduces to about 85L/hr. for the continuous operation. The smaller scale results from the higher effective utilisation of the continuous operation compared to batch.

Looking at the overall cost of goods there is a reduction at the 500kg/yr. scale as one goes from the hybrid to the fully continuous scenario. This pattern is not seen when scaling up to the 2000kg/yr. case. In this case, the move to the perfusion bioreactor operation has a negative impact on CoG resulting in the hybrid operation being the more attractive. So what is happening? The CoG model provides that insight, the cost breakdown shown in figure 5 provides a clue. In the upstream perfusion operation, media costs dominate the CoG and as process scale increases raw material costs play a more dominant role in
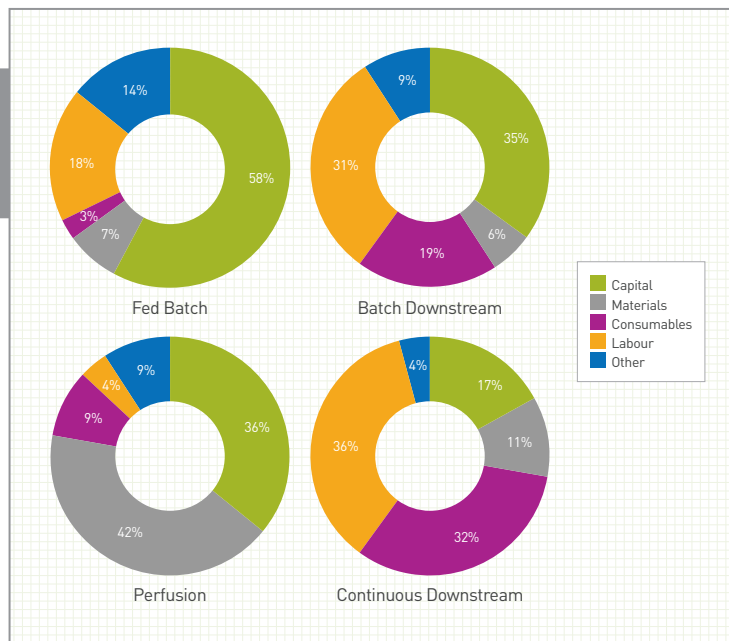
overall CoG. Therefore when looking at perfusion media consumption, product titre and media costs are important cost drivers when comparing this approach to fed batch operation.

	500 kg/yr				2000 kg/yr			
	Capital 10^6		CoG $/g		Capital 10^6		CoG $/g	
Batch								
Fed Batch bioreactor	72.6	58%	31.3	33%	93.4	53%	11.1	28%
Batch DSP	52.8	42%	63.7	67%	83.2	47%	28.2	72%
Total	125.4	100%	0%	95.0	100%	0%	176.6	100%	0%	39.3	100%	0%
Hybrid								
Fed Batch bioreactor	72.6	76%	31.3	35%	93.4	76%	11.1	33%
Continuous DSP	23.2	24%	57.0	65%	29.4	24%	22.5	67%
Total	95.8	100%	24%	88.3	100%	7%	122.8	100%	30%	33.6	100%	15%
Continuous								
Perfusion bioreactor	21.4	47%	24.4	29%	33.7	51%	16.7	42%
Continuous DSP	24.3	53%	58.8	71%	32.2	49%	23.3	58%
Total	45.7	100%	64%	83.2	100%	12%	65.9	100%	63%	40.0	100%	-2%

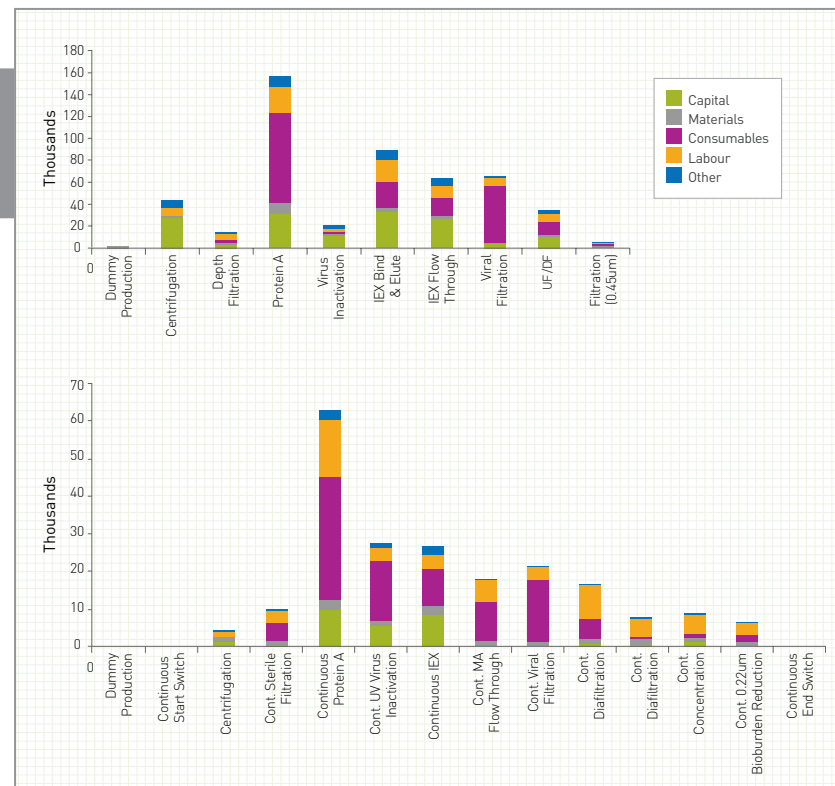Fig. 4: Comparison of cost of goods across the scenarios considered.

Looking at the overall downstream costs, we see again a reduction at the 500kg/yr. scale when moving from batch to continuous operation of around 8%. However as we increase scale to 2000kg/yr. the CoG reduction is larger at about 20% of the DSP operating costs. Looking into the detail of the cost distribution for both formats, the Protein A unit operation cost is a dominant cost driver. In the breakdown of the downstream costs we see that there are key differences between the batch and continuous processes. If the continuous processes represent a more heavily utilised asset we would expect the consumables and material costs to dominate and capital to diminish when compared the batch process. In figure 5 we see this effect. What is surprising is that there is no real reduction in the proportion of the labor costs. In assessing labor costs a conservative approach has been taken to assigning labor to operations. In reality the expectation for highly automated operations is for the labor component to be lower giving scope for further savings in this area.

Fig. 5

Cost breakdown for batch and continuous operations for manufacture at 500kg/yr scale

In this limited example of using a scalable cost model, we have shown that modelling the different technologies provides valuable insight into the key cost drivers. This allows the user to identify potential areas of savings and examine the dynamics of scale. Although this is only one example with a two scale of operations, it is nevertheless possible to develop insights that highlight the potential of continuous technology. Based on this study, we can draw the following conclusions about continuous processing:

- Lower capital inputs
- Upstream needs to be considered separately from downstream
- Upstream cost benefits (batch vs. perfusion) depend on media volume requirements, media costs, product titre
- For downstream savings are seen with more costs moving to variable costs such as materials, consumables

There is much more work required to better understand the impact of continuous technology and to optimise its use. The shift of fixed costs (capital) to operational costs is important in terms of operational flexibility and responsiveness. The full benefit of this is not captured in traditional CoG modelling; further work is needed using NPV analysis to quantify this. Using the more sophisticated modelling approach afforded by BSP, we can identify potential cost and operational benefits for current and future manufacturing.

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1)   BioSolve Process and proprietary process modelling package by Biopharm Services Ltd

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Dr. Timothy Johnson is an Associate Director in the Commercial Cell Culture Development department at Genzyme, A Sanofi Company, in Framingham MA. He received a Ph.D. in Chemical Engineering from the University of Washington, Seattle in 1999. While at Genzyme Corp., Tim has been responsible for supporting, understanding, and improving the production of commercial cell culture processes; for bioreactor process development, optimization, scale-up, scale-down, and technology transfer. Most recently, he is the technical lead for developing and scaling-up Genzyme's continuous manufacturing platform for the universal production of biopharmaceuticals.

10

Vision: Integrating Upstream and Downstream in a Fully Continuous Facility

Introduction

As the biotechnology industry continues its maturation, it is now faced with challenges from its own successes. Examples include increasing competition and associated concerns with speed to market, increasingly diversified product portfolios that include stable products (e.g. antibody) and complex, less stable products (e.g. recombinant enzymes), along with high and low product volume demands. Additionally, current companies require rapid adjustment of production capacity to accommodate fluctuating market demands (Kamarck, 2006). These challenges can be further complicated by the concept of regional manufacturing throughout the globe. Moreover, there is a growing focus on product batch-to-batch and site-to-site product quality and consistency partly due to enhanced analytical techniques as well as regulatory oversight. It is the author's perspective that one potential solution that collectively addresses these diverse concerns lies in the conversion of traditional batch manufacturing to that of a compressed, integrated, and continuous model.

Process intensification through conversion from batch to continuous manufacturing has long been applied in other industries, including steel casting (Tanner, 1998), petrochemical, chemical, food and pharmaceutical (Reay et al., 2008; Anderson, 2001; Thomas, 2008; Fletcher, 2010; Laird, 2007). Despite the differences between these industries, the advantages of continuous manufacturing are always the same, including steady state operation, smaller equipment size, higher volumetric productivities, streamlined process flows, low cycle times, and reduced capital costs (Utterback, 1994).

Currently, there are two dominant platforms for biopharmaceutical manufacturing: (1) perfusion bioreactors, typically used for production of less stable proteins (Fig. 1, Panel A), and (2) fed-batch bioreactors for production of stable proteins, such as MAbs (Panel B). In both cases, the bioreactor operation

is followed by multiple batch unit operations, including clarification, capture, polishing chromatography and hold steps. The continuous capture technology discussed by Warikoo et al. (2012), when integrated with upstream reactors allows for a significantly streamlined process train (Panel C) due to elimination of non-value-added hold steps, dramatically shorter residence and cycle times, reduction of equipment size, and overall facility minimization. For example, when high producing clones and robust chemically defined media are utilized, this platform can achieve very high cell densities and volumetric productivities while operating at steady state. As a result, sufficient production capacity can be achieved with smaller bioreactors ($\leftarrow$500L) vs. traditional processes where reactor scales may exceed 10,000L. The use of cell separation devices that simultaneously clarify the harvest of cells and cell debris eliminates the traditional clarification unit operation. Most importantly, the direct integration of the continuous capture step makes harvest hold tanks obsolete, and replaces the large batch capture column with up to 2 orders-of-magnitude smaller columns used in the continuous system. Furthermore, continuous processing of the harvest confers significant advantages with respect to protein quality. Specifically, elimination of the harvest and other hold steps decreases target protein exposure to enzymatic, chemical, and physical degradation and thereby mitigates product stability risks. With these objectives in mind, additional corporations have begun to pursue variations of their own continuous processing platform (Daszkowski, T.)

Fig. 1

Schematic of traditional and future manufacturing platforms for perfusion bioreactor process. (A) Traditional perfusion manufacturing process; (B) Traditional 10-20kL fed-batch manufacturing process; (C) New integrated continuous manufacturing platform.

## Critical Systems for Continuous Production

Upstream Systems

The success of upstream continuous perfusion reactor operations for commercial production has been reported throughout the literature. While traditional stainless steel reactor systems are an industry mainstay, progress in the development and robustness of single use bioreactors provides for an attractive alternative. Most notable vendors in the single use arena are Sartorius, Hyclone, and Xcellerex, and which are capable of supplying reactors in excess of 1000L. While reactor type and vendor tends to be a matter of corporate and process preference, several cell separation devices also exist upon which to facilitate continuous operations. Systems in use by major corporations include inclined plate settlers (Biotechnology Solutions), tangential flow filtration (various vendors), alternating tangential flow filtration (Refine Technology), acoustic resonance (BioSep – Applikon Biotechonology), and centrifugation (Centritech – Pneumatic Scale Angelus). Each system will have its various pros and cons that should be considered, especially the need for additional clarification should incomplete cell separation result.

Downstream Systems

Several continuous chromatography systems have been made available by Novasep (Pompey, France), Tarpon (Worcester, MA), Semba (Madison, WI), Massimo Morbidelli (Zurich, Switzerland), and GE Healthcare (Piscataway, NJ), which open up novel opportunities for the implementation of the integrated continuous bioprocessing concept. For an in-depth discussion of the periodic counter-current (PCC) chromatography (GE Healthcare) methodology, see Warikoo et al. (2012). While there are numerous technical features of these systems that will affect long-term, robust performance, the most critical may be the ability to functionally close the systems and protect them from adventitious agents throughout the process duration. To this end, some of the available systems provide options for incorporating gamma irradiated disposables and/or traditional stainless steel fabrication with steam sterilization methods.
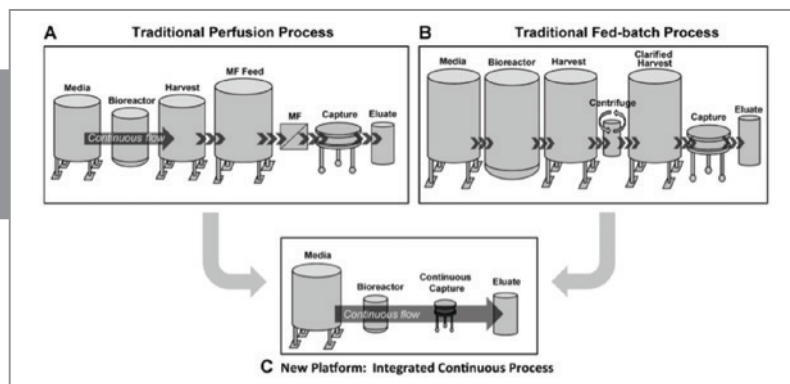
Integrated Continuous Bioprocessing

Integrated continuous bioprocessing is a novel solution that offers unique advantages over traditional approaches for recombinant protein manufacturing. This new platform has been successfully applied at development scale to drugs with diverse properties, such as a high-volume stable protein (MAb) and a low-volume less stable protein (rhEnzyme), which define the boundaries of real-world production scenarios (Warikoo et al. 2012). At large scale, the successful implementation of the platform requires a functionally closed system that can be maintained free from foreign organisms for prolonged periods of time.

Our vision of the biomanufacturing "facility of the future" based on the integrated continuous platform is outlined in figure 2. This general floor plan utilizes multiple parallel and independent continuous production lines designed as a functionally closed system that offers multi-product and multi-purpose manufacturing capability with reduced room classifications. The flexibility of this scheme enables rapid increase or decrease of production capacity based on real-time market demand using a "numbering up" approach rather than the traditional volumetric scale up. The flexibility of the system may be further enhanced by incorporating disposable solutions, both upstream and downstream. As the equipment footprint is dramatically smaller, the size of the required manufacturing facility and the related capital cost are significantly reduced (↗50%). This reduction in size, and cost, also facilitates the ability to have the pilot and clinical-scale manufacturing process at the same scale as final production, therefore nearly eliminating technical and timeline risks traditionally associated with technology transfer and scale-up. Additional advantages of this platform are that Large- or small-volume drugs, and the production of either stable or unstable proteins can be achieved while operating at a high level of standardization and mobility, thus facilitating the visionary concept of decentralized and portable regional manufacturing throughout the globe.

While the proof-of-concept demonstration of continuous biopharmaceutical manufacturing focused on continuous operations from media feed through to product capture (Warikoo et al. 2012), there have already been significant advances towards continuous processing through to drug substance (Konstantinov, K. (2013), Daszkowski, T. (2013)). These ideas for continuous

bioprocessing are taking hold throughout the industry. As such, it is expected that numerous conceptual and pilot designs will be unveiled in the near future with various degrees of continuous cadence suitable to the strategies associated with the respective corporations. While the respective unit operations may differ slightly, the core concepts outlined here will most likely remain throughout the various designs.
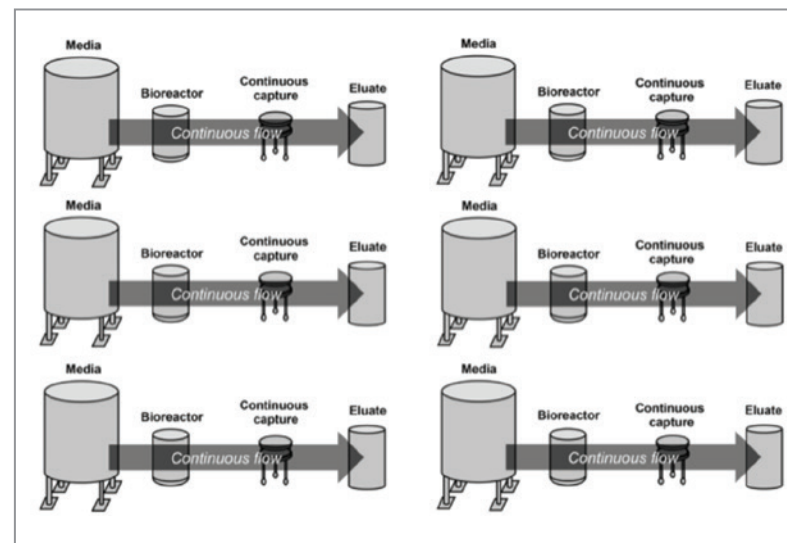

Fig. 2

General floor plan of a multi-product biomanufacturing facility utilizing the integrated continuous bioprocessing platform, implemented as six independent and parallel process trains. The key facility design concepts are: functionally closed systems, modularity, small equipment footprint, flexibility, "numbering up" instead of scale up.

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