Advanced Technologies in Biopharmaceutical Processing

An increasing number of pharmaceuticals in human and veterinary medicine are being developed using advanced genetic and other methods that focus on modification of somatic and embryonic cells. These methods, in the setting of drug manufacture, call for new processes that go beyond the traditional unit operations of chemical and biological production, such as batch submerged culture.

This volume explains how technologies developing in the last decade function in producing advanced biopharmaceuticals, such as hormones, cytokines, therapeutic enzymes, modified proteins, and transgenic products, to name a few. From large-scale animal cell bioreactors to patient-customized products, this volume describes the effects of new technologies on biopharmaceutical processes and guides users in how to apply new technologies in process development.

From the Foreword

"...provides a comprehensive approach to biopharmaceutical manufacturing that has been sorely needed.... This book provides an anthology of information in essentially all areas of bioprocessing, from genomics to final fill and finish, while it weaves the crucial elements of regulatory compliance throughout each step in the process."

—Keith L. Carson, Chairman and Founder,
The Williamsburg BioProcessing Foundation

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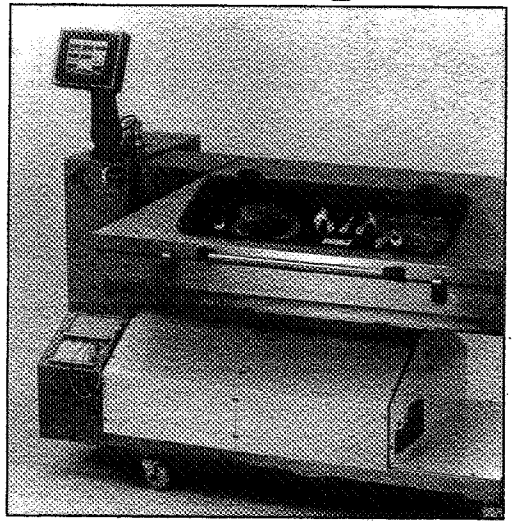

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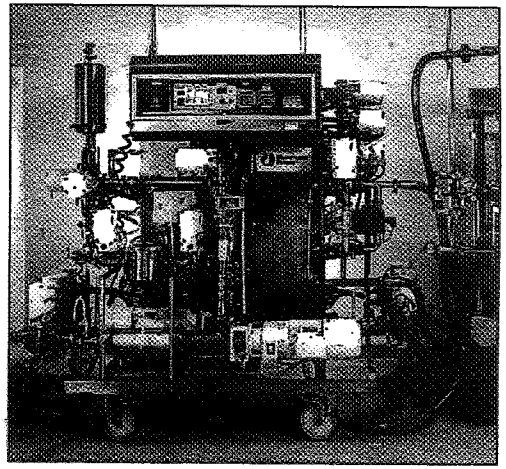

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4

NEW TECHNOLOGIES IN BIOPHARMACEUTICAL UPSTREAM PROCESSING

J. Dowd, G. Cosentino, S-B. Chao

4.1 Introduction

Large-scale production of recombinant biopharmaceutics was first accomplished through the culture of genetically manipulated bacteria. The technology for their efficient growth in large bioreactors was well established and, in relative terms, these microorganisms were the simplest to genetically modify. However, not all biologics lend themselves easily to synthesis in prokaryotes. Limitations in size and complexity of the product (especially as it relates to glycosylation and folding) quickly led investigators to consider a broader range of expression systems. Simple eukaryotes such as yeast and fungi, as well as insect cells, were successfully evaluated for their ability to synthesize a variety of more complex proteins. But the characteristic glycosylation pattern of recombinant polypeptides prepared from these systems was often suboptimal, because non-human carbohydrate structures were frequently incorporated and could negatively impact the clinical efficacy or safety of the derived biotherapeutic. It was recognized that higher-order eukaryotic

cells derived from mammalian species offered the advantages of appropriate post-translational modifications as well as proper folding, assembly, and secretion of the complex protein structures. Drawbacks of mammalian cell culture included a requirement for more sophisticated manipulations to introduce heterologous genes, complex nutritional requirements, long doubling times, low rates of protein synthesis, and the relative sensitivity of the cell to physicochemical stressors.

In spite of these processing challenges, nearly two-thirds of recently licensed biotherapeutics in the US and EU are derived through large-scale mammalian cell culture (Walsh 2005; Chu and Robinson 2001). Furthermore, products manufactured via mammalian cell culture processes represented 64% of the market value for the top ten recombinant therapeutic proteins sold by 2003 (Pavlou and Reichert 2004). Due to the advantages of product quality, it is expected that the trend toward mammalian cell culture will continue for the foreseeable future. Many of the new technologies developed for upstream biopharmaceutical processing have served to address the specific challenges of mammalian cell culture. Selections of these new developments are described here. A variety of general reviews on this topic are available (Hu and Aunins 1997; Hesse and Wagner 2000; Chu and Robinson 2001; Marks 2003; Campbell 2004; Coco-Martin 2004; Hilton 2004; Wurm 2004; Butler 2005).

4.2 Facilities

The design, construction, commissioning and qualification of biopharmaceutical facilities have been challenging for manufacturers, engineering firms, and equipment suppliers. To compete in the global market, these facilities must comply not only with GMP requirements worldwide, but also with local codes, laws, and regulations. The development of new technologies, such as Process Analytical Technology (PAT) (www.fda.gov/cder/OPS/PAT.htm), the use of disposable equipment, and the use of advanced automation technology will affect the design and operation of biopharmaceutical and vaccine facilities.

The cost of biopharmaceutical facilities has been rising in the last decade. There are multiple factors that affect the cost of bringing these facilities on-line. The following is a partial list:

- Due to historical limitations in analytical technologies and an incomplete understanding of the relationship between process variables and final product quality, biopharmaceutical processes have historically been viewed as "black boxes." The prevailing view is that the process equals the product. The view is reinforced by conservative regulatory approaches. The hurdle to get approval for the biopharmaceutical products is much higher than the "well characterized" small-molecule–based pharmaceuticals. This leads to a much higher cost to bring the biopharmaceutical products to market.
- There is as yet no harmonized regulation regarding the facility and process for biopharmaceuticals. Companies have to design facilities to meet most stringent requirements.
- Companies need to get quick facility approval at all costs, which leads to overspending to remove any potential difficulties during Pre-Approval Inspection (PAI).
- Considerable amount of money is spent on non-value-added cosmetic features rather than the protection of the product. Examples include mirror finishes on stainless steel equipment and facilities and classified spaces (clean rooms) though they are not needed (e.g., closed processes).
- Confusion regarding required process water quality often leads to process water being overspecified, without economic or scientific justification.

The advances in processing technology, such as sterile tube welding, Cleaning-In-Place (CIP), and Sterilizing-In-Place (SIP) have made it possible to use less classified space for the upstream process. The industry, as advocated through organizations such as the International Society for Pharmaceutical Engineering (ISPE) and the Parenteral Drug Association (PDA), is working slowly with the regulatory agencies to clarify the confusion and to provide guidance for the facility, utility, and process design. Some new industry trends in facility design and operations are discussed next.

4.2.1 Process Areas Classification

A typical biopharmaceutical facility has four basic process areas:

- Medium and buffer preparation
- Upstream area
- Downstream area
- Utility and mechanical system areas

Facilities are also divided into "live" and "non-live" areas. For most facilities, the Upstream area is "live,"while all other areas are "non-live." When the product is a living organism (such as many vaccines), the "live" area may extend to the Downstream portion of the process, including final fill and finish operations.

The required classification for each area is the basis for facility design and qualification. A summary of the classifications for a typical biopharmaceutical manufacturing facility is shown in Table 4.1.

Generally, controlled non-classified (CNC) rooms are the minimal requirement for closed processes. Most manufacturers choose to upgrade

Table 4.1 Classifications for Key Process Areas

Area	Classification (ISO)*	Classification (EU)*	Classification (US)*
Utility and Mechanical Areas		Controlled access but non-classified	Controlled access but non-classified
Medium and Buffer Prep	Class 8	C	100,000
BioSafety Hood	Class 5	A	100
Seed Lab	Class 7 or 8	B or C	100,000
Fermentation	Class 8	C or D**	100,000**
Clarification	Class 8	C	100,000
Purification	Class 8	C	100,000
Final Purification	Class 7	B	10,000
Final Filtration of the Drug Substance	Class 5	A**	100**

*A more detailed definition of ISO, US, and EU classification can be found in the FDA Guideline on Sterile Drug Products by Aseptic Processing and European Commission Annex 1, Manufacture of Sterile Medicinal Products.

**A lower classification can be used if the operation of the process is conducted in a closed system or an isolator.

air classifications, even where processes are closed, to provide secondary segregation and ensure that product exposed accidentally will not be at risk. It is general practice to tighten the control of the environmental conditions as the process moves from fermentation through clarification to final purification and filtration.

4.2.2 *Process Water*

Water is an important component of all processing solutions. The quality of the water will depend on the recombinant system used, the phase of manufacture, and the intended use of the product. The acceptable grade of water will depend heavily on the stage at which it is to be used during manufacture, the subsequent processing steps, and the nature of the final product. It is essential that a water quality specification is chosen that has no deleterious effect on cultures, products, and their production. Water for Injection (WFI) must be used in the final formulation of biopharmaceutics. For any other steps in the production, Purified Water, Highly Purified Water (HPW), or WFI may be used. Typical water quality requirements are shown in Table 4.2. Over specification of water quality may be used to simplify

Table 4.2 Typical Water Quality Requirements for Biopharmaceutical Manufacturing

Type of Manufacture	Product Requirements	Minimum Acceptable Water Quality
Synthesis of all intermediates of APIs prior to final isolation and purification steps	No requirement for sterility or apyrogenicity in API or the pharmaceutical product in which it will be used	Potable Water*
Fermentation media	No requirement for sterility or apyrogenicity in API or the pharmaceutical product in which it will be used	Potable Water*
Final isolation and purification	No requirement for sterility or apyrogenicity in API or the pharmaceutical product in which it will be used	Potable Water*
Final isolation and purification	API is not sterile, but is intended for use in a sterile, non-parenteral product	Purified Water
Final isolation and purification	API is sterile and not intended for parenteral use	Purified Water
Final isolation and purification	API is not sterile, but is intended for use in a sterile, parenteral product	Purified Water with an endotoxin limit of 0.25EU/ml and control of specified organisms
Final isolation and purification	API is sterile and apyrogenic	Water for Injections

*Note: The table lists the minimum required quality for water used in biopharmaceutical API manufacturing if water quality does not have a major impact on the process and product. Companies often build a higher quality water system to provide flexibility of the facility.

supply systems or to increase facility flexibility, but this practice can sometimes lead to increased cost of production.

4.2.3 *Controlled Temperature Rooms and Storage Rooms*

The intermediates and final products are often stored under refrigerated conditions (typically 2–8°C) or frozen, because most biopharmaceutical products are heat-sensitive. Also, it may be necessary to perform some process steps in cold or warm rooms (e.g., incubation rooms). Special design considerations must be given to controlled temperature rooms with classified air requirements. Clean room classifications of the area housing controlled temperature rooms must be adhered to. In addition to normal clean-room environmental monitoring (EM), these areas must be continuously monitored for temperature and relative humidity control. During start-up, these rooms must also be qualified and validated for intended use.

4.2.4 *Bioreactor Size*

There has been a trend in the industry to move to larger bioreactors for cell culture-based processes. Economy of scale is an important factor in determining the cost of manufacturing. However, it is also important to note the initial investment in a typical facility with 12,000-liter bioreactors is in the range of $500 million to $1 billion dollars (US). In addition, any process failure, which may result in product rejects, can cost tens of millions of dollars per batch. Fortunately, advances in process control and monitoring have enabled the mitigation of equipment and facility failure. Alternative cultural modes (fed, batch, perfusion), which are gaining acceptance, can be used to reduce required bioreactor size. Bioreactors should be sized depending on the market demand for the products, yield of the process, mode of operation, and other techno-economic factors. Life Cycle Costing (LCC) should be routinely employed (Dutton and Fox 2006) to aid in process option decisions.

4.3 Media

It is highly desirable, bordering on being a regulatory requirement, to have a medium formulation that is free of animal-derived components (ADCF) (www.fda.gov/cber/bse/over.htm). Medium optimization involves amino acid concentrations, as well as vitamins and salts. The optimization is also dependent on the process option, for example, fed-batch or perfusion operating modes. Hence, medium optimization can be challenging, especially because positive selection is often complicated by the noise in the system. In the rush to develop a process, there is often little opportunity to optimize medium components. Consequently, this is usually done in a second phase of optimization. Design of Experiment (DOE) software, such as those that employ fractional factorial design, mixture design, Pontryagin's optimization, genetic algorithms, or central composite design, can be used to facilitate optimization (See www.jmp.com, www.minitab.com, www.mathcad.com, www.umetrics.com).

Each process option will have different endpoints. For example, fed-batch cultures may employ a "deep" medium formulation, minimizing the concentrations of salts, while maximizing amino acid concentrations. This may have the desired effect of extending culture viability by delaying the accumulation of inhibitory by-products. A perfusion culture may be designed to minimize costs, especially when high volumes are being processed.

4.3.1 *Animal Derived Component Free Medium Formulations*

Hydrolysates can be a useful adjunct for free amino acid compositions because these amino acids may exist as n-mers (peptides), which may lead to greater stability, such as in the case of glutamine with L-alanyl-glutamine and L-glycyl-L-glutamine dipeptide (Stehle et al. 1982). Often a performance test is needed for QC lot release, however, which may be a simple growth assay tested under standard conditions. Some components (e.g., purified amino acids) can be derived from an animal source; however, regulatory agencies require full sourcing history of all defined medium components (www.fda.gov/cber/bse/over.htm). New plant (soy, wheat, barley) or synthetic sources of these components are available (www.jrhbio.com, www.sigmaaldrich.com, www.hyclone.com).

4.4 Cell Banking and Inoculum Train

The origin of the master cell bank (MCB) needs to be well described and appropriately maintained for future production (http://www.fda.gov/cder/guidance/ichq5b.pdf). The MCB considerations largely deal with the genetic construct. The working cell bank (WCB) is derived by expansion of the MCB, with key considerations being the methods, reagents, cell age, and storage conditions. Typically, cryopreservatives such as DMSO or PEG and selection agents such as methotrexate are included in the cell bank formulation. These inhibitory components necessitate significant post-thaw dilution.

The cells are quickly frozen and thawed using blast freeze/thaw technology (www.thermo.com, www.fosterrefrigerator.co.uk). Typically, the MWCB is frozen as a 1 mL "bullet,"of 10 to 50 million cells. The inoculum train starts with this frozen bullet. Newer technologies employ larger EVA cryo-bags (www.stedim.com) to maintain the MWCB in larger volumes, but with similar cell concentrations. This has the advantage of speeding the time to production

process by 7–10 days. For animal cell culture the 50- or 100-mL EVA cryo-bags enable direct inoculation into a bioreactor, as opposed to (T-flask) expansion. The health of the culture used to generate the MWCB is key to the speed of recovery during the inoculum train.

In general, culture volume is increased threefold to fivefold with each passage of cells through the inoculum train. This operation continues until the required volume is reached for seeding the production bioreactor. This requires a total of 2–4 weeks and up to 10 passage steps. At a small scale (e.g., < 20 liter working volume), the culture is commonly maintained in simple glass or disposable plastic vessels (i.e., shake flasks, roller bottles, spinner flasks) that are held at constant temperature, agitation speed, and headspace CO_2 concentration (for pH control). Agitation is controlled via orbital shaker, mechanical roller, or magnetic stirrer, while temperature and CO_2 are controlled passively by operating the unit in a defined environment. At volumes generally above 20 liters, the culture is scaled up in batch mode through fully equipped bioreactors with independent control loops for temperature, pH, dissolved oxygen, and agitation.

Significant capital investment is required to install separate bioreactors of appropriate size to support each linear step of the inoculum train. To reduce such costs, variations on the scale-up strategy are often practiced. One such example is to seed the bioreactor at the minimal working volume that can be supported by the design of the equipment (i.e., lowest placement of probes and impeller). Following appropriate cell growth, the culture is diluted back to the seeding density by adding fresh medium to the maximum working volume of the bioreactor. Such fed-batch "top-up" strategies reduce hardware costs and provide greater flexibility for operations in multi-product/multi-process facilities. Another approach is to operate the seed bioreactor in perfusion mode by feeding fresh medium to the culture and using a retention device to increase biomass to high cell density. In this way, bioreactor scale-up ratios as large as 25-fold could be used in successive steps for expanding the inoculum train.

A wide variety of novel disposable bioreactor systems have been introduced in the last decade, and these systems will have an increasing impact on the future design of preculture strategies for biomanufacturing processes. Examples of such systems include the Wave Bioreactor™ (Wave Biotech; www.wavebiotech.com), AppliFlex (Applikon; www.applikon-bio.com), FibraStage (New Brunswick Scientific; www.nbsc.com), and Cell Factory (Nunc; www.nuncbrand.com). In Figure 4.1, the Wave Bioreactor™ system is illustrated. A series of base units designed to accommodate culture volumes from 100 milliliters to 500 liters are available in this product line. All process stream contact surfaces of such systems are single-use, eliminating the need for development and validation of cleaning protocols normally

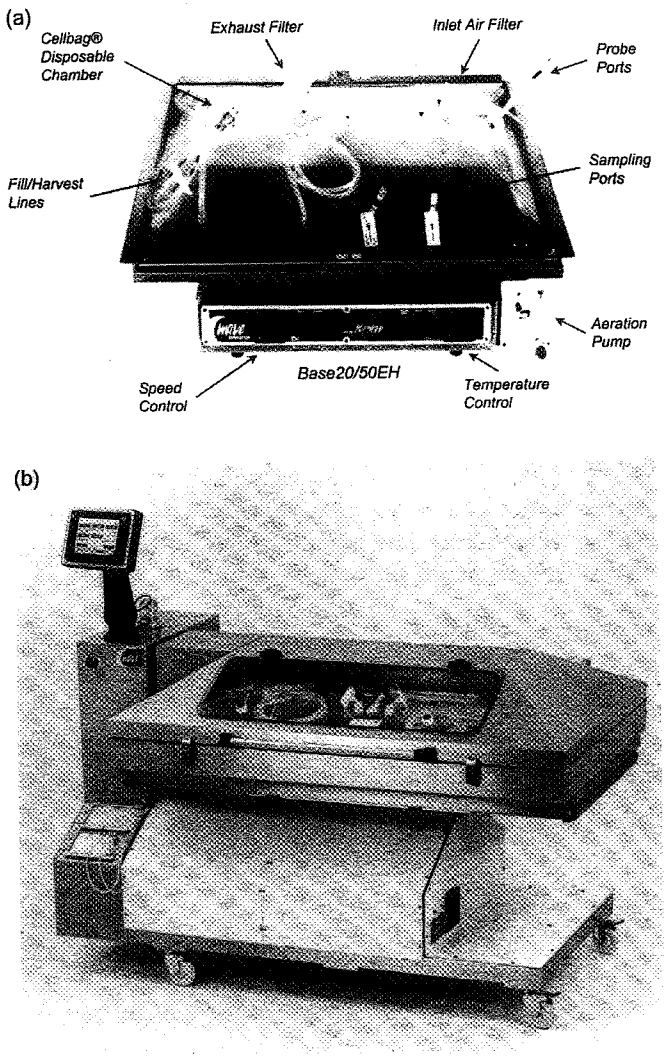

Figure 4.1: The Wave Bioreactor™ system (photos courtesy of Wave Biotech)
Top: 20/50EH base unit with 20L cellbag (10L working volume)
Bottom: 200EH base unit with 200L cellbag (100L working volume).

executed between runs. However, the requirement to carefully evaluate extractables/leachables from disposable systems intended for GMP use should not be underestimated (Hardy and Priester 2004). The design of the disposable bag system can also significantly reduce the number of open manipulation steps required for cell passaging, thereby limiting the risk of contamination during scale-up of the inoculum train.

4.5 Production

4.5.1 *Suspension and Anchorage-Dependent Systems*

Recently developed processes for manufacture of recombinant therapeutic proteins are based mainly on cell lines adapted to suspension culture. Suspension systems—characterized as a freely homogenous mixture of cell mass, soluble nutrients, metabolites, and secreted product—are favored because of simplified scale-up, monitoring, and control, and because of the availability of extensive bioengineering experience from several decades of large-scale microbial fermentation.

Nevertheless, not all commercially relevant cell lines are amenable to growth in suspension. Anchorage-dependent cell lines require a compatible substratum on which to attach and replicate, and a number of such cell lines (i.e., WI-38, MRC-5, Vero) are presently used for recombinant protein or whole virus vaccine production. Simple small-scale culture systems for anchorage-dependent culture provide a flat attachment surface over which sufficient volume of medium is layered for immersion of the cells (i.e., petri dish, T-flask, roller bottle, and commercial units such as the Nunc Cell Factory or Corning CellCube). These systems tend to suffer from a low available surface area to volume ratio and practical complexities regarding their scale-up potential. Microcarrier technology has resolved some of these issues through the use of small beads (100–200 μm in diameter) derived from silica, glass, dextran, collagen, polystyrene, or other materials for the attachment surface (Griffiths 2001). This approach improves the surface area to volume ratio, which allows for higher achievable cell densities compared to static monolayer systems. Microcarrier culture can be executed in mechanically agitated bioreactors, providing the same advantages of well-defined scale-up, improved monitoring, and efficient process control as provided for suspension systems. However, microcarrier culture is typically more sensitive to localized shear forces generated by microscopic eddies in close proximity to the beads (Papoutsakis 1991; Gregoriades et al. 2000). Scale-up of microcarrier culture should take this sensitivity well into account, since such mechanical shear can significantly reduce culture viability.

Macroporous microcarriers, such as Cytopore (GE Healthcare, www.microcarrier.nu) or CultiSpher (Percell Biolytica, www.percell.se), allow for the immobilization of either anchorage-dependent or suspension adapted cell lines. The open sponge-like structure of the macroporous microcarrier permits cell access and attachment to the interior of the bead, thereby providing a larger surface area per unit volume and subsequently higher potential cell densities. This design also circumvents some of the shear issues, because the cells are sheltered inside the structure of the microcarrier. This additional protection allows for use of higher agitation and aeration rates so that elevated bead loadings may be employed upon scale-up. It is possible that cells attached more deeply within the porous structure can experience anoxic stress, thereby limiting the viability of the culture (Preissmann et al. 1997). However, although growth may be reduced, overall expression yields for recombinant proteins may be higher in macroporous microcarriers than in traditional suspension culture systems (Spearman et al. 2005).

Disposal of spent microcarriers is a major drawback of these systems. Disposal methods are often manual, requiring significant time and labor. Central disposal systems are prone to plugging by the microcarriers. Also, complete removal from bioreactors can be difficult.

4.5.2 *Overview of Batch and Continuous Processing*

Suspension culture can be run in either batch or continuous mode. For batch processes, cells are seeded into the production medium and are grown until either the depletion of the limiting nutrients or the accumulation of toxic metabolites causes a rapid drop in viability and productivity of the system. The cells and accumulating product are exposed to a constantly changing environment under these conditions. Fed-batch culture is a commonly used variation of batch processing that extends the productive lifetime of the culture and leads to substantial improvements in cell density and product titer. This is accomplished through an optimized feeding strategy, which supplements the medium with key nutrients at defined time points during the process.

Continuous culturing systems differ from batch processing in that nutrients are continuously fed to the bioreactor while a harvest stream containing product and metabolites is removed at an identical rate. Advantages of such an approach include more consistent product quality due to a stable culture environment, removal of toxic cellular metabolites, reduced risk of product degradation, and more effective use of the facility's production capacity. Continuous culturing without cell retention (i.e., chemostat or turbidostat mode) is well described for microbial organisms (cf. Shuler and Kargi 2002); however, lower inherent growth rates makes this an impractical option for mammalian cell culture. Perfusion systems have successfully addressed this restriction through the use of cell retention strategies that allow for continuous culture at high flow rates while maintaining elevated cell density in the bioreactor.

The relative merits of fed-batch versus perfusion strategies continue to be actively discussed within the bioprocessing community (Marquis et al. 1990; Werner et al. 1992; Kadouri and Spier 1997; Cacciuttolo 2005; Ozturk 2005). While the final choice can only be made in light of specific process characteristics (cell line, medium, and product) as well as the nature of the

manufacturing facility, some general practical comments on this topic are offered here:

- Productivity as a function of bioreactor volume is higher in continuous culture, thereby reducing capital outlay and indirect operating costs for the facility. However, productivity per unit volume of processed media is generally higher for fed-batch operations, thereby reducing raw material and fluid handling costs.
- Fed-batch processes tend to be more easily automated and controlled.
- Improved recovery of unstable, inhibitory, or poorly expressed proteins can be achieved using continuous perfusion processes, which allow for constant removal and stabilization of secreted product from the bioreactor. A protein is said to be unstable if it has an activity half-life in the culture broth that is comparable to the culture period. It is therefore desirable to separate the protein of interest from the culture broth and transfer it to a stable matrix as quickly as possible.

4.5.3 *Fed-Batch Operation*

A typical fed-batch scenario involves the addition of a concentrated nutrient feed to extend the culture time, allowing the cells to produce more protein. The batch medium is formulated, typically through optimization studies (see www.americanlaboratory.com and www.nbsc.com for automation) that will give high titers and, in general, high cell numbers. At the end of the batch phase, prior to the cells entering stationary phase, programmed concentrate additions are made to allow for further cell growth, or to maintain cell viability. The concentrate could be as simple as an addition of glucose or a combination of specific nutrients. Alternatively, a stoichiometric (Xie and Wang 1994a,b) approach can be applied, with a view to supplying those nutrients that have been depleted from the culture. The goal of the fed-batch operating mode is to sustain a constant environment for cellular productivity.

In animal cell culture, much work has been done with glucose and glutamine, which are the main conventional energy sources for mammalian cell culture, as limiting nutrients (Zielke et al. 1978). The additions range from a simple bolus addition to a continuous feed based on feed-forward and feed-backward information. The feed-backward control may be accomplished through sensor-based measurements of cell number, glucose concentration, dissolved oxygen concentration, and so on. Alternatively, a feed-forward arrangement may be used, employing a model-based schedule. A typical modeling index is the integrated viable cell hours (Dutton et al. 1998), and the extension of this index relative to batch operation has been correlated with increases in protein titers. Regardless, the goal is to maintain conditions that will maximize culture viability, allowing for additional protein production.

Case Study 4.1: Protein Productivity as a Function of Cell Hours

In batch culture mode, most data, including secreted protein titer, is obtained on a cumulative basis. Cell concentration is an exception, being obtained on an instantaneous basis. Although the production of protein is obviously a function of the viable cell concentration, it is difficult to discern the exact relationship quantitatively from a comparison of the primary data (Figure 4.2a).

In fact, the protein concentration is not directly connected to the number of viable cells in the medium but to the number of cumulative hours that cells have been in the medium producing protein. Similar to labor hours, the cumulative volumetric cell hour, CH_{Vol}, can be obtained by integrating the raw viable cell concentration data, defined as:

$$CH_{Vol} = \int_0^t X_V \, dt \tag{4.1}$$

where:

CH_{Vol} = volumetric cell hours
X_V = the viable cell concentration
t = time

A simple numerical integration technique utilizes the natural log average of each pair of viable cell concentration values at adjacent sampling points (Dutton et al. 1998):

$$CH_{Vol} = \sum_0^t \frac{(X_{V_{t+\Delta t}} - X_{V_t})}{\ln(X_{V_{t+\Delta t}} | - X_{V_t})} * (t_{t+\Delta t} - t_t) \tag{4.2}$$

where:

t_t, $t_{t+\Delta t}$ = sampling times ($t_{t+\Delta t} \leq t$)
Δt = time period between consecutive samples

When the protein concentration is plotted against the cumulative volumetric cell hours, the relationship is more readily apparent (Figure 4.2b), and the exact correlation can be quantified: the cell specific protein productivity, q_p, is the slope of the plot of protein titer against cell hours (Figure 4.2c). In this example, protein production is partially growth phase-associated with an increase in productivity during the late declining-growth phase.

A typical example of fed-batch culture application has been the production of monoclonal antibodies (Mabs) by hybridomas. Cell specific productivity is not necessarily related to the specific growth rate, likely because Mab specific productivity is not completely cell cycle phase-associated. For example, it has been reported that cells growing slowly at 32°C have significantly higher

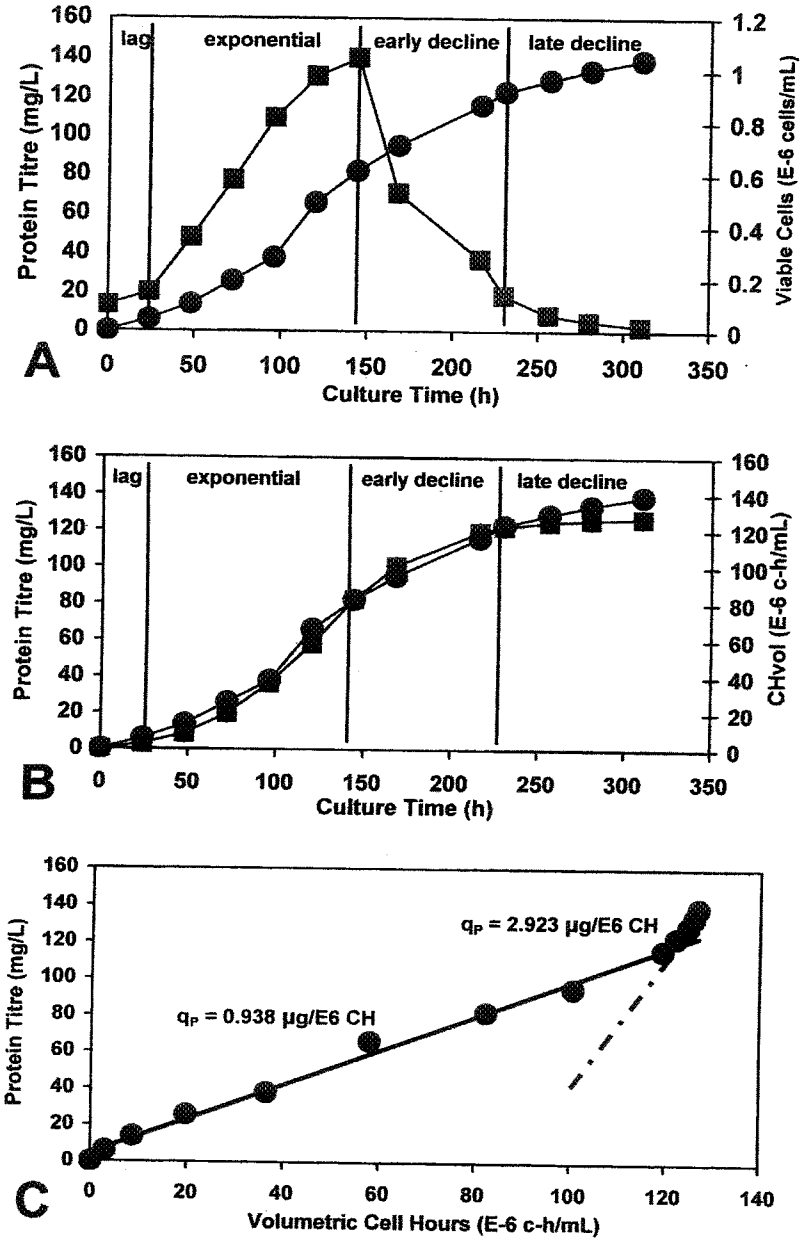

Figure 4.2: Protein productivity by mammalian cells is a function of volumetric cell hours (CH_{Vol}).

A) Protein titre and cell population during a batch culture.

B) The same batch culture with the cell density recalculated as volumetric cell hours.

C) Protein titre shown as a clear function of volumetric cell hours.

● = protein titre; ■ = cell concentration; q_P = specific protein productivity.

productivity than cells growing faster at 37°C (Fox et al. 2005). Lowering the growth temperature from 37°C to 32°C causes cell cycle phase arrest in G0/G1 phase.

It has been shown recently that non-replicating cells continue to produce the recombinant protein product, albeit at a lower rate than slow growing cells in the S cell cycle phase. Indeed, cell specific productivity of recombinant proteins can extend well beyond the growth phase (Lloyd et al. 2000) into the decline phase where cells are accumulating in G1 prior to entering apoptosis (Dutton et al. 1998). Optimization of the cell culture process by adaptive model-based control has been shown to be dependent on suppression of apoptosis (Frahm et al. 2003).

Due to its simplicity and straightforward implementation, dissolved oxygen-based control (DO-stat) is often employed in aerobic industrial fermentations to schedule nutrient feeding in fed-batch operations (Yamane and Shimizu 1984). The objective of the methodology is to maintain the dissolved oxygen concentration at a more or less constant level above its critical value by manipulating the feed rate. The first step in the application of the DO-stat is the establishment of the critical oxygen concentration of the organism. The critical oxygen concentration is defined as the minimum oxygen concentration needed to maintain fully aerobic metabolism. This concentration is a characteristic of the microorganism in question and ranges usually between 0.1 and 1 mg/L O_2. Then, the oxygen concentration set point is established through experimentation at some concentration above the critical value.

In fed-batch operations, the key substrate feed is usually the energy source whose catabolism is directly linked to oxygen consumption. The exhaustion of the substrate causes the dissolved oxygen concentration in the medium to rise. Fresh substrate feed is pumped into the bioreactor when the dissolved oxygen concentration rises above the set point. Since the oxygen concentration profile in the fermentor is often noisy, it may require smoothing by a time series (moving average) filter to avoid any chatter of the feed pump. In principle, the DO-stat is an on-off controller. A drawback of the DO-stat fed-batch system is that any inadvertent medium overfeed can cause the oxygen concentration to fall below the critical value. Also, excess glucose feed in many yeast and microbial cultures is known to cause a switch to anaerobic metabolism even at relatively high oxygen concentrations (Pasteur effect). This could result in suboptimal operation with the microorganism's growth pattern tending to linear, rather than the more desirable exponential, during the fed-batch operation (Nor et al. 2001). Attempts to improve fed-batch performance have included hyperbaric oxygen supply (Belo et al. 2003), feed-forward exponential feeding based on oxygen consumption (Nor et al. 2001), and the use of two different dissolved oxygen set points with optimized bandwidths to turn the feed pump on and off (Lee et al. 2003).

4.5.3.1 Concentrate Formulation

A stable stock solution is key to successful fed-batch operation. Some feed components may not be stable in a complete concentrate formulation. Isotonic formulations (300–350 mOsm/kg) at neutral pH are required for the culture, and some medium components may not be stable under these conditions. An approach has been to formulate the amino acid portion of the cocktail under acidic conditions, vitamins under basic conditions, and salts and glucose under neutral conditions. Glutamine exhibits a first order abiotic decay forming ammonium with time especially in the absence of serum (Ozturk and Palsson 1990; Grossie et al. 1993), which can be deleterious to cell growth and may affect correct protein glycosylation and sialation (Cecchelli et al. 1983; Yang and Butler 2000). For this reason glutamine is often formulated separately, with the stock solution stored frozen and assigned a relatively short shelf life. Lipids can be formulated in ethanol, to ensure solubility at high concentrations. A small amount of ethanol is generally not a concern for the culture, as dilution is significant. Upon mixing, the medium concentrate should have a close to neutral pH value.

4.5.3.2 Optimization and Stoichiometric Feeding

Reproducibility can be improved by on-line monitoring and control, as compared to the traditional bolus type of feeding strategy. On-line monitoring provides a better estimation of timing and scale of the feeding based on cell population need. This is nevertheless challenging, because similar culture behavior can occur with variable nutrient levels. Recent developments in capacitance probes as well as multi-wavelength and fluorescent sensors allow on-line estimates of cell number and nutrient levels. However, not only are these measurements indirect, but they may not correlate to productivity. NOVA Biomedical (www.novabiomedical.com) offers new analytical BioProfile systems for extensive at time off-line analysis of culture broth. It may be possible to couple these technologies to arrive at a robust and reliable monitoring and control system. Significant validation hurdles must be overcome before these systems can be employed in the GMP setting.

4.5.4 Continuous Perfusion Culture

Continuous perfusion culture, in suspension or microcarrier operations, is well established and has been implemented for commercial production of many biotherapeutic proteins (Table 4.3).

The composition of feed medium and its rate of addition are critical during continuous processing and must be carefully optimized to balance specific

Table 4.3 A Sampling of Commercial Perfusion Processes

Name	Company	Cell Line	Reference
Advate	Baxter	CHO	www.advate.com
Aldurazyme	Genzyme/BioMarin	CHO	Rader, 2004
Fabrazyme	Genzyme	CHO	www.fabrazyme.com
Kogenate	Bayer	BHK	Boedeker, 2001
NovoSeven	Novo Nordisk	BHK	Rader, 2004
ProstaScint	Cytogen	Hybridoma	Rader, 2004
Rebif	Pfizer/Serono	CHO	Houlton, 2005
ReFacto	Wyeth	CHO	Boedeker, 2001
Remicade	Centocor	Sp2/0	www.fda.gov
ReoPro	Centocor/Lilly	Sp2/0	Rader, 2004
Simulect	Novartis	Myeloma	www.fda.gov
Xigris	Lilly	HEK	www.fda.gov

cellular requirements. Because perfusion culture is typically grown to high cell density, process feed-backward is generally on-line to ensure that this balance is maintained. The control output is the perfusion feed medium flow rate.

Table 4.4 summarizes some commonly utilized control loops in perfusion culture. There are several potential approaches to obtain process information, the most important of which provide an indication of cell concentration (Dowd et al. 2003) or an overall cellular rate of consumption (Dowd et al. 2001). The culture level in the vessel may be maintained using a low-level control loop, triggering the outflow of spent medium to the harvest container. Cell purging or bleed is also carried out to maintain the culture in a viable and active state by continuous removal of cells and particulate cellular debris as a predetermined fraction of the perfusion rate (Dalm et al. 2004; Swaving 2005). This is done to flush nonviable material from the bioreactor and to maintain the viable cell density at a steady-state value determined to be optimal for the medium composition and other process conditions.

A variety of cell retention devices have been employed to maintain elevated viable cell densities during perfusion culture. These can be loosely categorized as filtration, settling, or ultrasonic devices (reviewed in Castilho and Medronho 2002; Voisard et al. 2003). Barrier-based devices such as filtration suffer from clogging over time, especially at high cell densities in protein-free media common to modern bioprocesses. To address this limitation, dynamic systems are used whereby relative motion between the filter and the bulk culture generates shear at the filter surface and reduces fouling. Examples of this strategy include spinfilter and tangential-flow filtration (TFF) devices. TFF systems can be operated with nominal pore sizes of 0.1 to 0.5 μm, and therefore

Table 4.4 Control Loops for Perfusion Culture

Control Loop	Set Point	Input (Process Information)	Output (Manipulated Variable)
Perfusion feed rate	Cell specific value[1]	Aber capacitance probe, CEDEX, ViCell, Trypan blue counting	Medium pump speed
		Oxygen uptake rate (OUR)	
	Set feeding regime	Culture time or cell number	
Cell purge rate	Cell concentration	Aber capacitance probe, CEDEX, ViCell, Trypan blue counting	Purge pump speed (or on/off)
		Oxygen uptake rate (OUR)	
Vessel level	Vessel weight	Load cell, level probe	Harvest pump speed (or on/off)
Foam	Little or no foam	Visual observation	Anti-foam addition pump (operator required to close loop)

[1]Obtained through optimization studies (e.g., 0.3 nL/cell/day as in Dowd et al. 2003).

provide essentially cell-free perfusate that needs no further clarification. However, the tangential velocity of the culture fluid across the membrane surface is limited by the shear sensitivity of the cells; membrane fouling can thus still be an issue. Pulsing the feed across the membrane surface and changing directionality of the flow is an approach that has been shown to reduce fouling, and this strategy has been employed in a low-shear hollow fiber design, known as an alternating tangential flow or ATF system™ (Refine Technologies; www.refinetech.com). It has recently been reported that continuous perfusion of the robust PER.C6® production cell line with the ATF system allows for generation of extreme cell densities of more than 100 × 106 viable cells/mL, resulting in steady-state product titres similar to those achieved for optimized fed-batch processes (Yallop 2005; Swaving 2005).

Settling and acoustic devices do not use a physical barrier to retain cells in the bioreactor; therefore, fouling of these systems is much less of an issue. Settling devices are based on density differences between viable cells and culture fluid. Gravity settlers and centrifugation systems are members of this category. Countercurrent gravity settlers are relatively simple systems, usually comprised of multiple inclined plates that serve to reduce the linear velocity of upwardly pumped culture fluid to less than that of the sedimentation velocity of viable cells. Cells then accumulate in the lower settling zone and are recycled to the bioreactor while cell-free harvest is recovered from the top. At least one perfusion process based on a gravity settler has been commercialized (Joeris 2005), but long residence times and subsequent exposure of cells to suboptimal culture conditions can be a major issue for this technique. Continuous centrifugation devices (i.e., Westfalia, www.westfalia-separator.com, Kendro, www.kendro.com) serve to increase the settling velocity of the cells in a controllable manner and are therefore more flexible, particularly for optimization of large-scale perfusion processes (i.e., greater than 1,000L working volume). The complexity, reliability, and cost of sterilizable centrifuge equipment may be an issue and should be evaluated on a case-by-case basis.

Acoustic (ultrasonic) separation devices (i.e., AppliSens BioSep, www.applisens.com) serve to increase the sedimentation velocity of cells by inducing their aggregation in a plane-standing wave generated by a high-frequency acoustic resonance field. The loosely aggregated cells settle out of the acoustic field and are immediately disaggregated upon recirculation to the bioreactor. A number of parameters must be optimized for efficient use of acoustic filter technology, including cycle time, flow rate, acoustic power, recirculation, backflush frequency, and temperature control (Crowley 2004; Shirgaonkar et al. 2004; Gorenflo et al. 2005). Acoustic filter devices capable of separating cells at perfusion rates up to 250 L/day are presently available, and units that can accommodate 1,000 L/day are presently in development.

A frequently raised concern for continuous processing is the ability to create and maintain aseptic conditions over extended culture periods. However, the design of modern equipment and ancillary systems has minimized such risks and resulted in numerous successful examples of such operating strategies. Presterilized disposable bag/filter assemblies and sterile connecting devices that are increasingly used for media and other inputs to the culture further simplify the application of continuous processes on the manufacturing floor.

4.6 Scale-Up

The ultimate scale requirements are dependent on the stage of clinical development and the expectation of market size. Variables to consider when scaling up include temperature and oxygen transfer. Heat load needs to be considered and compact heat exchangers (www.exergy.com) can be used to raise the temperature of incoming fluids. The surface area to volume ratio in larger reactors is smaller, requiring higher wall temperatures to effect the

same heat transfer rates, but higher wall temperatures may damage cells. The alternative is to have longer equilibration times to reach target temperature. It is a common practice to operate the bioreactor at a suboptimal temperature, such as 34°C, during production.

Oxygen transfer rate is often used as a basis for scale-up design calculations. Sensitive mass flow controllers, using the input of the oxygen sensors as well as the controller output (direct measure of flow rates), allow for closing the material balance on the oxygen in the reactor. With a few simplifying assumptions about constant mass transfer coefficients and constant cell specific uptake rates, process information about cell density can be inferred. A simple mass balance for dissolved oxygen gives:

$$\frac{dC}{dt} = -OUR \cdot X + K_L a \cdot (C^* - C) \tag{4.3}$$

where:

C = concentration of oxygen,
C^* = interfacial oxygen concentration,
$K_L a$ = oxygen transfer coefficient,
OUR = oxygen uptake rate, and,
X = cell concentration.

These on-line cell concentration estimates provide a useful tool for bioreactor control.

Traditionally, parameters such as pH, dissolved oxygen, and temperature are monitored to gain better process knowledge and control. Also, it is important to measure biological parameters, and on-line measurements can indicate the physiological state of the process. In either fed-batch or perfusion modes, the actual marker for many of the key decisions during the process is the live cell concentration.

Of the available biomass assays, only the Aber™ Biomass Monitor (based on radio-frequency impedance) gives an on-line indication of the viable cell volume rather than the total number of cells (www.aber-instruments.com). Coulter counters (www.coulter.com) provide an off-line histogram of cell count and gate distinct (i.e., live versus small diameter debris) cell populations. The Cedex (www.innovatis.com) system automates traditional Trypan blue staining and counting, also giving an off-line measure of cell count and viability. Capacitance is practically the only on-line method available for cells growing either in suspension (especially as aggregates) or attached/immobilized to micro-carriers, often at high cell densities. On-line monitoring systems such as microscopy, multi-channel, and fluorescence sensors are currently under development and should be available in the near future.

In the last several years, systems biology has emerged as the interface between process systems engineering (PSE) and biology, with an overall emphasis on analyzing the complexity in biological systems using integrative systems approaches. Developments in genomics, proteomics, metabolomics, and bioinformatics are enabling a systems biology approach to process design, optimization, and control. For example, Principal Component Regression (PCR), coupled with other statistical tools such as Partial Least Squares (PLS) or Multivariate Analysis (MVA), is a powerful method for improving the understanding of metabolic fluxes in bioreactor systems. This analytical technique can be utilized to understand what may be the key factors for scale-up and medium optimization.

PLS and PCR are data analysis tools for finding relationships between variables. Variables are defined as either response variables (Y matrix) or predictor variables (X matrix). Both PLS and PCR are particularly useful when the number of predictor variables (columns of X) exceed the number of observations (rows of X) and the significant predictor variables need to be identified from the available set. To accomplish this, PCR extracts factors from the Y'Y and X'X matrices, while PLS, which is an extension of multivariate analysis, extracts factors from the Y'XX'Y matrix (Geladi and Kowalski 1986). Partial Least Squares regression has been proven to give a "better fit" and appears to be more suited for scientific applications than PCR (Phatak and de Hoog 2002). Partial Least Squares software is available in high-level programming frameworks, for example, MATLAB® or SAS®, which are convenient to use. Alternatively, efficient PLS computing algorithms such as NIPALS or SIMPLS (de Jong 1993) can be employed in a programming language of choice.

Case Study 4.2: Nutritional Profiling and Medium Design for Fed-Batch Culture

This case study pertains to Hybridoma 130-8F producing IGg type monoclonal antibody (Mab). The task at hand was to establish relationships between growth rate and IgG productivity (response variables) and the amino acid fluxes (predictor variables). Both the response and predictor variables were normalized as per "volumetric cell hours," CH_{Vol}.

To accomplish normalization, the concentrations of biomass, Mab, glucose, lactate, ammonia, and the amino acids were plotted against CH_{Vol}, and the average slope of the plot during the exponential growth phase was taken as the representative flux. A typical plot of Mab concentration as a function of the volumetric cell hours is shown in Figure 4.3. The slope of the curve was the Mab response flux used in the assessment. It is important to note that the Mab flux extended well beyond the exponential growth phase virtually unchanged (i.e., constant slope), indicating the non-growth–associated

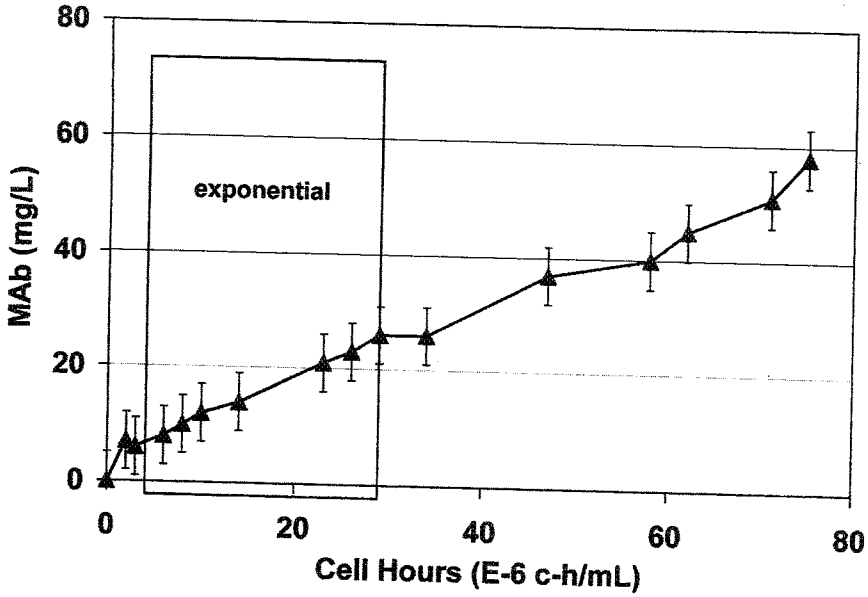

Figure 4.3: Hybridoma 130-8F grown in 0.5L batch spinner—Monoclonal Antibody concentration as a function of cumulative volumetric cell hours (Exponential Phase marked by box).

nature of Mab productivity. Non-growth–associated productivity, in turn, implied that Mab synthesis was a direct linear function of the G1 cell cycle phase and high productivity could be achieved by maintaining high cell viability, but not necessarily growth, in fed-batch culture. This, of course, required medium reformulation for the fed-batch operation.

Altogether, ten sets of batch observations were made using different glucose and amino acid concentrations. The specific growth rate in the exponential phase of growth (biomass flux) ranged from 0.0221 h^{-1} to 0.0456 h^{-1} (15.2 h to 31.4 h generation time) and cell-specific Mab productivity (Mab flux) ranged from 0.39 pg/106 cell-h to 1.03 pg/106 cell-h. The predictor variable matrix comprised 18 amino acid fluxes. The data space was analyzed by employing SIMPLS algorithm (de Jong 1993). Using successive extraction of predictor variables, the following amino acid fluxes were found to correlate with both Mab productivity and growth: glutamine, isoleucine, leucine, threonine, and valine. Consequently, the medium was tailored for fed-batch culture employing an amino acid cocktail enriched with these amino acids. However, any attempt to enrich the amino acid composition of full-strength growth medium to increase Mab production was negated by increased ammonia production, which negatively impacted cell viability. In fact, the five key amino acids also correlated positively with ammonia production. Diluting the feed medium, but increasing the relative concentrations of the key amino acids, was a successful means of alleviating ammonia toxicity in fed-batch mode. After the amino acid feed adjustments, Mab production was extended from 72 hours in batch culture to 160 hours in fed-batch culture without significant loss in viability (greater than 80% viability) or specific Mab productivity.

Case Study 4.3: Optimization of Cell-Specific Productivity

This study involved the optimization of tissue plasminogen activator (tPA) production from a CHO DUX-B11 cell line in perfusion culture using a systems biology approach. Glucose concentration was set as the index for medium utilization and modeling (Dowd et al. 2001; Handa-Corrigan et al. 1992; Hiller et al. 1993).

Using PCR and PLS analysis, cell-specific rates were investigated over a range of glucose set points in order to optimize operation and demonstrate proven acceptable ranges (PAR) and edge of failure (EOF). The cell-specific uptake/production rates were calculated as follows:

$$q_i = \frac{\left(\frac{(C_{i,2} - C_{i,1})}{(t_2 - t_1)} - \frac{F}{V}\cdot(C_{i,\mathrm{In}} - \bar{C}_i)\right)}{X_{V,LM}} \tag{4.4}$$

where:

q_i = cell-specific uptake/production rate for component i (pmol/cell day),
$C_{i,1}$ and $C_{i,2}$ = component concentrations at time points 1 and 2 (mM), respectively,
$C_{i,\mathrm{In}}$ and $\bar{C}_i$ = inlet and average component concentrations (mM), respectively,
t_1 and t_2 = time points 1 and 2 (days), respectively,
F = flow rate (L/day),
V = reactor volume (L), and,
$X_{V,LM}$ = log mean cell concentration (cells/L).

The effect of normalization is to similarly scale the cell specific rates, improving pattern detection (Geladi and Kowalski 1986). Here, the cell-specific rates were normalized using the following equation:

$$y_i = \frac{(q_i - \bar{q}_i)}{SD(q_i)} \tag{4.5}$$

where:

y_i = normalized cell-specific uptake/production rates,
$(q_i - \bar{q}_i)$ = average of the q_i values, and,
$SD(q_i)$ = standard deviation of the q_i values.

This transformation scales the values to a Gaussian distribution with zero mean. Decreasing uptake rates are negative numbers, while increasing uptake rates are positive, so that the patterns of metabolic uptake and production rates tend to mirror each other.

With decreasing glucose availability, the cell-specific rates of consumption or production of glucose and several other components declined. Glucose may thus be used as an indicator (Wold et al. 1987) for the pattern of

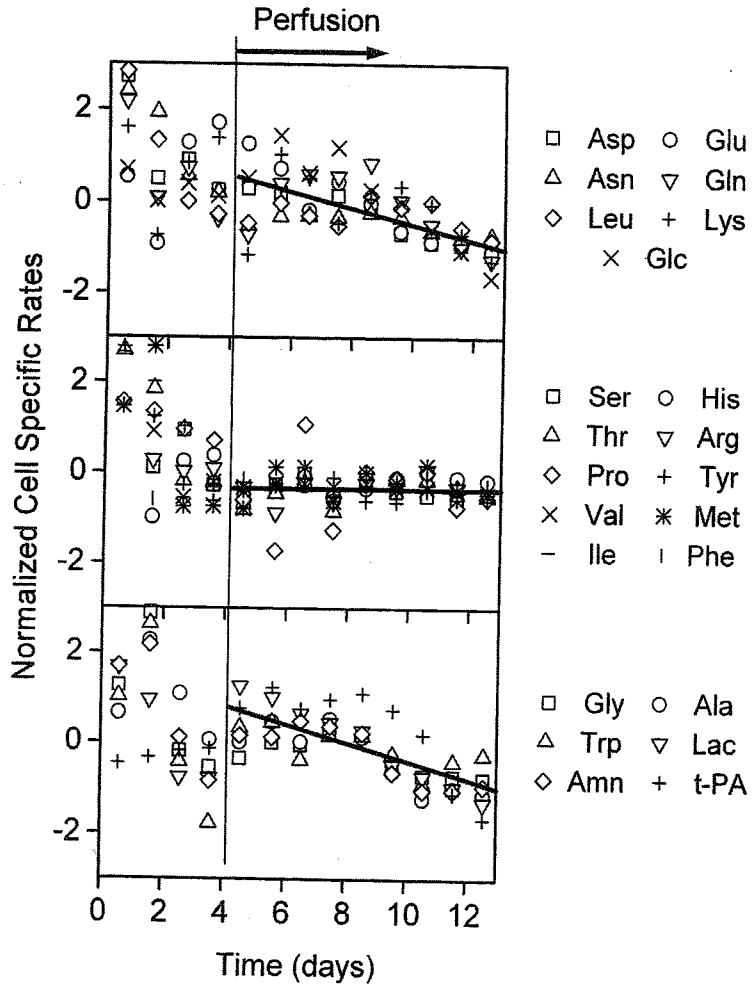

Figure 4.4: Normalized cell specific rates of uptake or production. The cell specific amino acid rates were normalized using Equation 4.5. For the period between 4 and 13 days, a number of amino acids exhibit a decline in cell specific uptake rates (top panel), or a decline in cell specific production rates (bottom panel). Another subset of amino acids exhibit no significant change in rate (middle panel).

utilization/production rates for all these substrates and metabolites, including protein (top and bottom panels in Figure 4.4, respectively).

Several medium components exhibit constant cell-specific rates as a function of medium depletion (middle panel of Figure 4.4). If process modeling was treated as a stoichiometric equation, the coefficients would be constant, regardless of the extent of medium utilization. Process modeling based on stoichiometry needs to include the significant effects of variable coefficients in assumed stoichiometric relationships, correlated with either medium or glucose depletion.

Glutamine and isoleucine are examples of the predominant patterns of amino acid depletion. These and some of the other amino acids exhibit linear relationships with increasing glucose depletion (Figure 4.4). Ammonium and alanine, by-products of glutamine metabolism, decreased with glucose concentration, indicating more efficient glutamine utilization under nutrient-limiting conditions. In agreement with the findings of Pelletier et al. (1994), ammonium production and glutamine depletion follow similar patterns for the batch stage (>15 mM glucose concentration), but tend to diverge in the perfusion culture at lower glucose concentrations.

As illustrated in Figure 4.5, small differences in the glucose set points (8.5 mM versus 6 mM) had a significant impact on the culture viability and protein titer. A transient peak in protein concentrations occurred at low glucose concentrations, while at slightly higher glucose concentrations, stable production was observed (Dowd et al. 2003). The protein production rate coincided with the uptake of several amino acids.

From these consistent (n = 8) observations, it appears that increasing the glucose depletion results in increased cell-specific protein productivity. However, this productivity increase was not sustainable, negatively affecting the viability after a few days and tPA quality as observed by sialic acid residue analysis (data not shown). This systems approach provided an indication of the biological activity in the reactor, which was later verified by off-line analytic results.

4.7 Scale-Down

Scaled-down models for the upstream process are important for troubleshooting, optimization, and execution of process validation procedures. This is particularly true for range studies within the Process Validation program. Typically, Process Validation requires consistent performance over three full-scale runs at set points (controlled parameters). Process Validation at scale would not be practical or economically feasible if parameter ranges were examined in full detail.

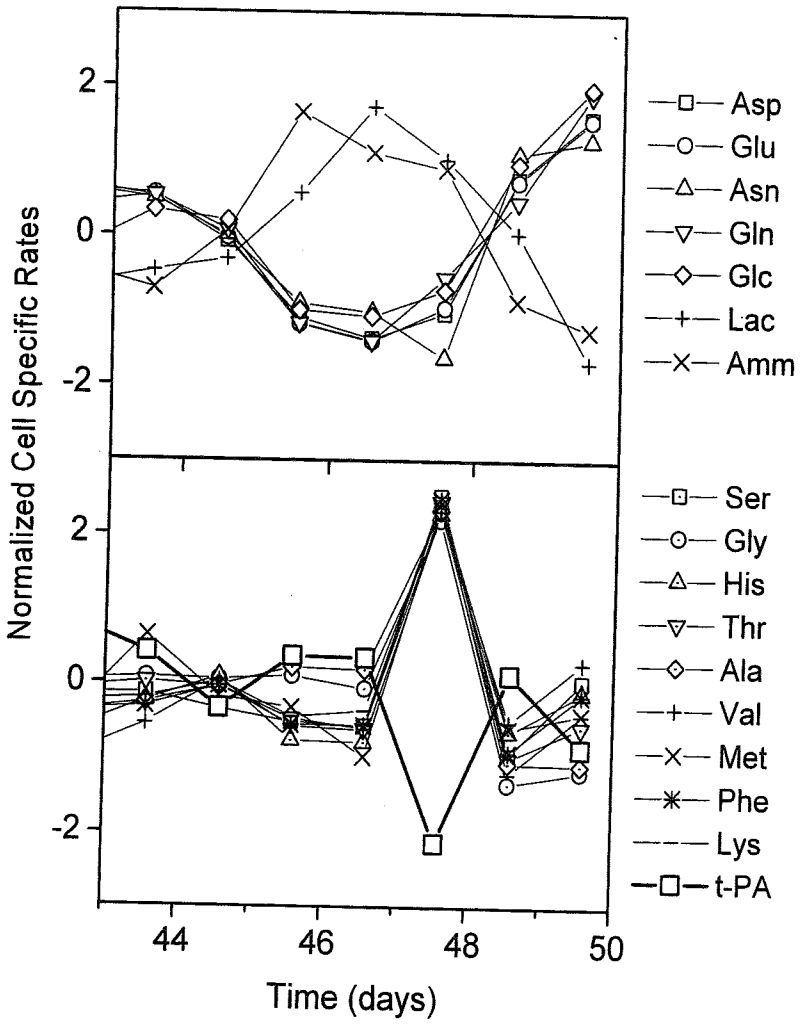

Figure 4.5: Normalized cell specific amino acid uptake and protein production. The cell specific amino acid uptake rates were normalized using Equation 4.5.

Efficient pre-validation studies provide the necessary background information to support Process Validation. Small-scale models also provide materials for assay development and qualification, and for prospective column re-use and viral clearance studies.

In a traditional sense, the goal of scale-down is to create a laboratory system that mimics performance of the parallel manufacturing process, both within and outside normal operating specifications (Rathore et al. 2005). A number of considerations must be kept in mind for such an exercise. Many of these are intuitive—such as the need for similar relative bioreactor geometries, agitation systems, sparger design, power input, and a good understanding of additional features that will affect heat or mass transfer characteristics of the system. A number of bioreactor suppliers can provide expertise to assist in matching the characteristics of commercially available lab-scale equipment with existing production units. However, other process details are equally important, and effort should be made to ensure that these are also comparable between the scales—quality and preparation of raw materials, generational age of the seed culture, sample handling and analytical techniques, calibrated accuracy of probes and control loops, and operational characteristics of ancillary equipment such as relative hold-up volumes on pumps or connected cell-separation devices. Only after careful evaluation of these parameters will it become possible to successfully qualify the model as a valid representative of the full-scale process.

Extreme scale-down of cell culture has been proposed as a means of streamlining the optimization program in upstream process development (Qualitz 2005). Shake flasks and spinner flasks have been well characterized as simple agitated culture devices (Freyer et al. 2004; Kumar et al. 2004; Sucosky et al. 2004) and are used extensively to evaluate productivities of newly isolated clones or to test performance in parallel medium formulation studies. These types of studies are resource-intensive, both in time and materials, for growing and evaluating cells under the many interrelated parameters that are characteristic of modern cell culture technology. Robotics and other automated systems are emerging that will mitigate bottlenecks. A number of groups are presently evaluating the applicability of miniaturized systems for such multiparameter optimization of microbial and cell culture processes. Examples of such extreme scale-down systems include 5 mL working volume in 50 mL conical tubes (De Jesus et al. 2004), 3 mL working volume in spectrophotometer cuvettes (Kostov 2001; Qualitz 2005), and 250 ML working volume in a microtiter plate format using printed circuit board technology for temperature control, gas delivery, and optical density measurement (Maharbiz et al. 2004). Novel technologies for *in situ* monitoring in such extreme scale systems will also be required, and studies have been described for optical quantitation of pH, oxygen, and CO_2 concentrations (Liebsch et al. 2000; Harms et al. 2002). Commercial microbioreactor systems (i.e., BioProcessors SimCell; www.bioprocessors.com) are available that simulate batch, fed-batch and perfusion operation in 30–500 ML working volumes.

4.8 Harvesting and Concentrating

4.8.1 *Cell and Debris Removal*

The cellular debris in the culture harvest will be highly variable depending on the culture type and needs to be characterized for appropriate optimization, typically with scale-down filter models. If a filter train is used, the goal is to minimize costs, which roughly scales with the surface area of the filters. In order to minimize the filter surface area, a series of filters is generally used,

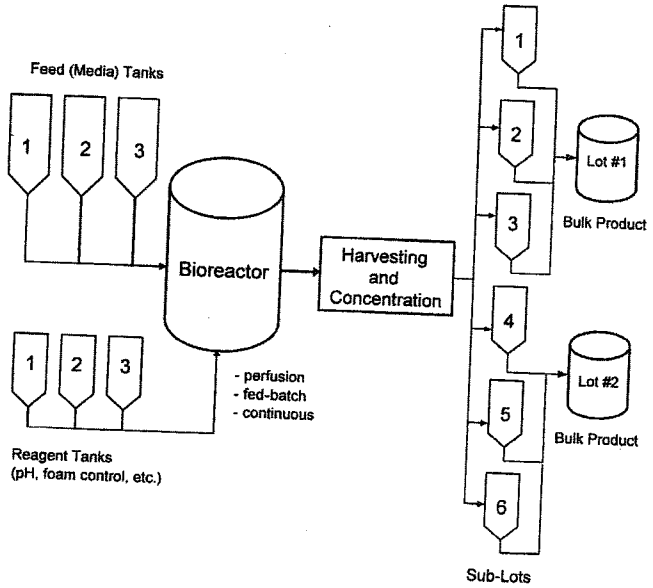

Figure 4.6: Biopharmaceutical Sub-Lots. In general a single batch bioreactor process equates to a single lot. When operating in fed-batch, continuous or perfusion mode, the distinction of a lot becomes blurred. Multiple vessels can be employed to harvest sub-lots, which can then be pooled into lots on the basis of specific criteria such as time, cell age, medium lot, etc.

with increasingly smaller pore size (see www.millipore.com). Alternatively, a centrifuge (www.westfalia-separator.com) may eliminate the large size portion of the distribution and a polish filter may be used to ensure that the particles are no larger than the target.

4.8.2 *Sub-Lots from Perfusion, Fed-Batch, and Continuous Culture*

In a continuous culture, the "lot" is typically defined as the bulk product formed following harvest and concentration of one or several sub-lots. Of course, all process components need to be tested, including not only the medium feed, but also sodium hydroxide for pH control, anti-foam C for foam control, and so on. Sterility testing of these supplies and similar process break points are often used to mark sub-lots. These inputs may be used "at risk," because analysis, particularly sterility verification, may not have been completed prior to use. In the event of a sterility breach, only the affected sub-lot is impacted. The emerging rapid methodologies for sterility testing may negate this concern in the near future. Careful documentation is required to capture the sub-lot composition at each bulk product break point.

4.9 Integration with Downstream Purification

The culture type and operating mode can profoundly impact downstream purification. The downstream process needs to be flexible enough to handle an increase in throughput, because cell clone, media, and so on can all be optimized to substantially increase the amount of protein. Indeed, there is the potential for cell culture processes to become "too efficient," because large-scale fermenters may produce protein to > 5 g/L levels (Butler 2005). It may be that the emphasis of future bioprocessing development will be in the downstream purification arena.

Some companies have taken the approach of performing an "isolation" of the protein, prior to downstream purification (i.e., direct capture of Mab from dilute harvest on a Protein A resin; cf. Remicade BLA reference #98-0012). These steps typically concentrate the protein. In addition, it is desirable to remove or reduce cell culture surfactants (such as Pluronic F-68 and Anti-foam C), which may interfere with downstream processes, and/or to change the protein matrix to stabilize the protein. Diafiltration is often employed to change the osmolality of the particle-free supernatant and can easily be combined with ultrafiltration-based concentration. Similarly, anion exchange chromatography can be used in a direct feed manner (Dowd et al. 2002) to stabilize a protein, using approaches and additives described in Chapter 6.

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5

NEW TECHNOLOGIES IN BIOPHARMACEUTICAL DOWNSTREAM PROCESSING

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5.1 Introduction

Within the biopharmaceutical industry, the term "Downstream Processing" is used to describe the series of unit operations that take a feed stream from an upstream process and produce a purified, bulk Active Pharmaceutical Ingredient (API). A typical downstream process will consist of the following steps: (1) initial clarification, (2) initial capture, (3) intermediate purification, and (4) final polishing. Depending on the physicochemical properties of the product and the nature of the contaminants present in the initial feed stream, the downstream process can be a straightforward, quick, robust procedure or a complex, time-consuming, and difficult multi-unit operation.

Although downstream processing procedures are highly variable, with constraints and demands largely defined by the production source, they all have some degree of commonality with respect to the overall purification strategy (Rathmore and Velayudham 2003). With few exceptions, all process strategies will start with an initial clarification to remove particulates and whole cells. Initial clarification is usually accomplished with a filtration-based technology, but centrifugation and fluidized bed options are also utilized.