

# Fed-Batch Mammalian Cell Culture in Bioproduction

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Originally developed for optimizing microbial fermentation, the fed-batch approach has become a leading technology in biologics production based on animal cell culture. For manufacturing-scale applications, we can simply address the batch, fed-batch, and perfusion operating modes (1). But as the number of basic reactor types and production modes/strategies grows, absolute categorization and terminology become more difficult. That is especially true when considering small scales and more academic (research) approaches, in which concepts such as “fed perfusion” have been described (2). Such references can be confusing to beginners trying to make sense of the basic approaches available.

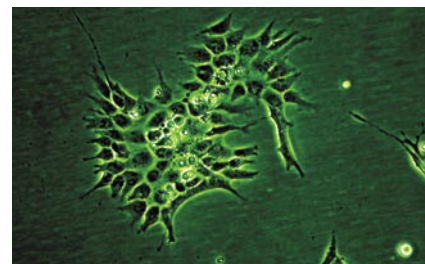
Regardless of the cell, product, or reactor addressed, fed-batch mode specifically refers to an approach in which a concentrated solution of nutrients is added at particular intervals, with no product harvested

until the end of the run. An early implementation of the fed-batch approach involved an additional charge of sucrose added in mid-process to a quiescent *Saccharomyces* fermentation. That feeding allowed for production of a key biological product: Champagne just wouldn't be the same without bubbles from the CO<sub>2</sub> produced by that sucrose feeding!

In fact, many of today's animal cell fed-batch approaches originate from microbial fermentation systems. For example, in early penicillin fermentation it was first thought that a relatively expensive sugar (lactose) was required for optimal production. It was later revealed that high production (over biomass) resulted from feeding with less expensive glucose at an appropriate rate. Current fed-batch systems for animal cell bioreactor production are based on adaptations and extensions of such earlier work.

## THE CASE FOR FED-BATCH

Many combinations of reactor types and culture modes are now available for use in bioproduction: e.g., rocking wave-agitated bag batch, stirred-tank or air-lift fed-batch, and hollow-fiber or spin-filter perfusion. Each presents distinct characteristics, as listed in the “Modes” box. No single production format is inherently superior; that determination depends on many manufacturing capabilities, requirements, and goals (Table 1). Those arise from the nature of each specific product, scale of production, and a manufacturer's total production schedule. Furthermore, there is



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ongoing evolution in

- available options (e.g., a single-use perfused hollow-fiber system for perfusion cultures from BioVest International, [www.biovest.com](http://www.biovest.com), and HyClone's single-use stirred-tank systems for batch cultures)
- underlying production demands and goals (e.g., heightened requirements for molecular quality and homogeneity of product)
- system component efficiencies (e.g., often 10- to 100-fold increases in cell and product yields).

The fed-batch version of stirred-tank culture has become most popular at large scales. The primary driver of this trend is obvious: Adding nutrients to a batch culture in mid-run can increase the quantity of product harvested. But the prevalence of fed-batch over other modes is due to many practical factors including reliability, ease of scalability, and application latitude (see the “Reasons” box). Efficiency of a particular production process can be measured several ways, and the fed-batch approach wins in many cases. Its greatest improvements derive from increases in the integral of viable cell concentration and volumetric productivity.

Of the culture processes proven valuable, debate over the best approach for large-scale production has evolved to that between fed-batch

**PRODUCT FOCUS:** ALL PRODUCTS OF MAMMALIAN CELL CULTURE

**PROCESS FOCUS:** PRODUCTION

**WHO SHOULD READ:** PROCESS DEVELOPMENT, MANUFACTURING, AND PROJECT MANAGEMENT

**KEYWORDS:** CELL CULTURE, FED-BATCH, OPTIMIZATION, PAT, MONITORING AND CONTROL, MEDIA AND SUPPLEMENTATION

**LEVEL:** INTERMEDIATE

and perfusion, most often in stirred-tank reactors (3). Perfusion advocates offered good theory supporting its use (4), and those large-scale manufacturers still using it show that it can be highly productive, especially in a manufacturing facility dedicated to very few products — or in producing a particularly labile product.

Fed-batch mode is easier to operate, so early results are faster and cheaper to obtain. It more readily lends itself to changing products (or cell types) within a given facility. And whereas the newest and most valuable engineering of a system is often held as a trade secret, it is more the case with perfusion processes. Because per-cell yields have dramatically improved of late (5), many of perfusion's previous advantages have been reduced. In fact, for products made by the newest ultrahigh-yield fed-batch systems (now exceeding 5 g/L), an argument can be made that for some product types there are now few advantages to perfusion.

Key advantages of fed-batch such as reduced direct costs (6) have remained or increased (e.g., reduced medium consumption, waste generation, and personnel requirements). Additional advantages include ease of process validation and characterization; reduced aggregate population doublings from master stocks; reduced footprint and turnaround time; greater lot consistency and definition; ease of downstream clarification, harvest concentration, and storage; and overall reduced time to product approval. Interest is growing in developing more closed-loop and disposable bioreactor technologies (7), and the simplicity of the fed-batch approach lends itself to those emerging technologies (8).

## CELL LINES

Suitable cell lines for bioproduction display certain common characteristics: an ability to grow, unclumped, in suspension culture; stable and productive integration of heterologous DNA; a capacity for desired posttranslational modifications; robust growth and high levels of production in a variety of media; and adaptability to a variety of

selection and production environments (9). Mammalian cells share many metabolic processes and display many similar characteristics, including in protein expression. However, some cell-line-specific differences can significantly affect performance in production. For example, glycosylation of a given protein can vary as expressed in various mammalian systems (10). And sometimes two production clones — even those derived from the same parent line — can display significant differences in metabolic requirements and production performance.

Although many mammalian cell lines have been used for commercial production of biologicals, most advanced large-scale fed-batch optimization has been performed for proteins, especially monoclonal antibody (MAb) pharmaceuticals. CHO, SP2/0, and NS0 have predominated, with significant promise held out for others such as HEK 293 and the PER.C6 cell line from Crucell NV ([www.crucell.com](http://www.crucell.com)).

The Chinese hamster ovary (CHO) cell line was established from a Chinese hamster's ovary biopsy over 40 years ago. Lately its use with dhfr and glutamine synthetase (GS) based selection is prevalent in bioproduction. SP2/0, a BALB/c mouse myeloma derived over 25 years ago, has an extensive record as a null parent for hybridomas and transfectomas. NS0, another BALB/c mouse myeloma, was isolated from the MOPC-21 derivative NS1 over 20 years ago and has been subcloned and otherwise modified at many locations. Currently popular are the ECACC #03061601 and a proprietary glutamine synthetase (GS) selection strain belonging to Lonza Group ([www.lonza.com](http://www.lonza.com)).

HEK 293 was derived from a human embryonic kidney in the late 1970s by transformation with adenovirus 5 DNA and found useful in producing recombinant adenovirus and adenoassociated viral vectors (rAAV). Recent developments in suspension culture and transient transfection techniques are promoting its use in producing a number of bioproducts — particularly large glycosylated human proteins. And the

## CELL CULTURE MODES

**Batch:** Initial medium charge provides all nutrition for the entire run.

**Continuous:** Prolonged feeding/harvest maintains a steady state.

**Dialysis:** Nutrients/metabolites are swapped across a membrane.

**Extraction:** A two-phase system lowers some toxic metabolites.

**Fed-Batch:** Concentrated nutrients/factors are added during the run.

**Filtration:** Media are changed and cells are retained through a (spin) filter.

**Perfusion:** Media are continuously exchanged (e.g., by gravity or centrifugation).

**Repeated:** A fraction of the biomass provides seed for the next cycle.

PER.C6 cell line was derived from a single healthy human retinal cell using rDNA (rather than viral) technology. Initially intended for production of virus-based products, it is now beginning to be applied in large-scale manufacturing of a wide range of biopharmaceuticals.

## BASAL CULTURE MEDIUM

Recent demands of economy, reproducibility, transportability, qualified raw material availability, and regulatory concerns have led to the development of protein-free, animal-derived-component free, and chemically defined cell culture media. Many existing products are made by procedures established in older, even serum-containing formats, but all the most popular cell lines mentioned above can now be cultured efficiently in these newer formulations (11). Some supplementation of a standard medium is often required to provide components that are too labile to include in basic formulations; that are optional to standard use; that support either cell-line- or application-specific requirements; or that are vector-determined selection agents (12).

Most clonal derivatives present their own metabolic phenotypes, so very large-scale producers face another issue: whether to use a commercially available medium or to invest in optimizing a proprietary formulation for a particular clone and/or production format. In batch mode processes, such optimization

can increase production levels two- to fivefold (13). Customized fortification of serum-free media (SFM) for batch culture has advanced to the point that in some cases amino-acid and carbohydrate concentrations in basal media are so high that most NaCl is omitted to maintain normal osmolarity. But in special large-scale production modes (especially fed-batch culture), beginning with a much leaner base medium can provide even greater efficiency. For example, although general metabolic pathways in mammalian respiration have been understood for decades, analyzing the practical consequences of glucose and glutamine feed timing and concentrations continues to be a profitable activity.

High initial levels of glucose can contribute to an early boost in culture expansion but also can induce many cultures to shift to a metabolism known as the “Crabtree effect.” These high levels of glucose inhibit oxidative metabolism resulting in production of lactate, which causes premature cellular stress or apoptosis. High concentrations of glutamine can contribute to ammonia levels exceeding 10 mM, which can impede not only cell growth, but also aspects of product formation such as protein glycosylation (14).

Some bioprocess scientists advocate the design of low-glucose and/or low-glutamine basal media, leaving those substrates to be introduced in metered doses as part of fed-batch “controlled nutrient feeding” (15). Related approaches (e.g., complementing with other metabolic cycle participants) have reportedly allowed cultures to detoxify themselves from some inhibitory metabolites and reduced accumulation of amino acid byproducts, thus increasing both culture and product quality (16). There are even reports of controlling the troublesome accumulation of CO<sub>2</sub> in large-scale reactors by manipulating the type and concentration of respiration-active carbohydrates.

The costs of such optimization efforts can be quite high depending on the facilities, expertise, and schedules available. And there are no guarantees: Some fed-batch cases show little improvement in yield. So

large-scale manufacturers commonly select one of many commercially available SFM and spend their optimization efforts instead on feed component composition and timing/rates of administration (17).

### FEED COMPONENT OPTIMIZATION

Some companies meet feeding requirements by simply adding concentrated solutions of commercial media or standard amino acids plus glucose and glutamine at midculture (18). Although generic feed mixtures are commercially available, most large-

scale manufacturers find that developing feed formulas customized to their particular cell lines, media, products, and/or bioreactor applications is well worth the effort (19,20) — as shown in Table 2. Many popular and powerful feeds depend on undefined or animal-based materials such as serum extracts and protein hydrolysates (21). Because chemically defined and animal-product-free media are becoming standard, however, such materials will not be addressed here.

Most feed solutions include high concentrations of materials in an

**Table 1:** Operating mode choice factors; these ratings are suggestions; some are subjective and vary according to product, scale, and specific reactor technology.

Factors to Consider	Batch	Fed-Batch	Perfusion	
			Hollow Fiber	Other
Cell generations through run	Low	Medium	High	High
Clone/product/materials latitude	High	Medium	Low	Low
Closed system capability	Yes	Yes	Yes	Yes
Disposable materials capability	Yes	Yes	Yes	Limited
Ease of automation	High	Medium	Medium	Low
Equipment/automation requirements	Low	Medium	High	High
Existing vs. available expertise	Easy	Medium	Hard	Hard
Failure risk (batch)	≤ 5%	≤ 5%	≤ 10%	≤ 10%
Footprint (total space consumption)	Low	Medium	High	High
Lot consistency	High	Medium	Low	Low
Lot size/definition	Fixed	Fixed	Varies/pooled	Varies/pooled
Materials, downstream steps, direct costs	Medium	Low	High	High
Multiproduct/clone production capability	High	Medium	Low	Low
New product institution turn-around time	Low	Medium	High	High
Occurrence in bioproduction facilities	Medium	High	Low	Low
Personnel (FTE) requirements	Low	Medium	High	High
Process control requirements/complexity	Low	Medium	High	High
Process development (time/cost)	Low	Medium	High	High
Product changeover/start-up	Easy	Medium	Medium	Hard
Product homogeneity	Medium	Medium	High	High
Product reactor-residence time	High	High	Low	Low
Production cycle time	Short	Medium	Long	Long
Productivity (by time/batch)	Low	Medium	High	High
Productivity (volumetric)	Medium	High	Low	Low
Raw harvest volume/storage/processing	Medium	Low	Low	High
Regulatory/quality constraints	Low	Medium	High	High
Scalability (capability/ease/cost)	Maximal	Maximal	Constrained	Constrained
System modeling ease (including scale-down)	High	Medium	Medium	Low
Technology transfer (between plants and to CMOs)	High	Medium	Low	Low
Throughput (product per reactor unit)	Low	Medium	High	High
Turnaround time	Low	Medium	Medium	High
Waste generation	Medium	Low	High	High

original medium empirically identified to be disproportionately consumed (see the “Feed Components” box). An important consideration in developing a feed solution is that, at the time it is administered, a culture may be at as much as 10 times its original cell density, which can allow some components to be brought to significantly higher molar levels than at culture seeding (22). More specific approaches in development include influencing (even controlling) particular cellular metabolic pathways or activities, such as feeding with nucleotide sugars or their precursors to enhance product glycosylation (23).

Although basal media must support a sparse (low cell density) culture’s viability and initial progression, more advanced feeds can be used to promote special pathways or cellular responses that could be detrimental in early stages. For example, special ingredients or material concentrations that increase protein secretion at the expense of cell division may be infeasible for basal media used to seed a culture but desirable for a culture at  $>3,000,000$  cells/mL. And culture conditions such as ambient pH, ammonia level, nutrient availability, and particular sugar complement can influence the type and degree of product glycosylation (24, 25), so many processes include feed components known to be active in those areas.

Development of a specialized and customized feed solution is an iterative process mainly involving spent medium analysis and resupplementation. Usually the first one or two rounds of such activity will supply the most benefit. Eventually no increase in cell mass or product will be obtained even when every measurably depleted component is restored. Depletion of measurable nutrients is not the only limiting factor in a cell culture system, which points to an advantage in applying the scale-down approach as much as possible here (see Process Development, below). Fully optimized feeds often exist as two or more separate solutions that support more than one rate of introduction and feed pH (e.g., for reasons of solubility) while protecting the physical integrity of special feeds

(e.g., high-concentration lipid dispersions).

Those who use a particular cell line for several different projects find that developing a “generic” feed for the parent (or null) line, in the particular basal media and reactor conditions generally used, improves productivity for all derived producer clones (26). However, even with such in-house systems, additional benefit is most always found in producer clone-specific optimization. The degree of benefit varies greatly and is affected by clone development/selection procedures, yet it can increase harvested product.

As with basal media, glutamine and glucose levels are a major factor in many feeds. Asparagine, arginine, and cysteine are among those amino acids that often have disproportionately high use rates. Others such as alanine, proline, and isoleucine, tend to vary greatly in use between cell lines or even individual clones. Some vitamins and metals provide significant benefits, and some can be tolerated at many times the levels found in commercial basal media.

Both the physical stability of a concentrated feed solution and the chemical stability of its components are significant concerns. Depending on the amount and  $pK_a$  of amino acid constituents, varying medium pH can drastically increase their solubility. Blending concentrates of previously solubilized individual components, rather than sequentially adding powders to one solution, can lead to a higher total concentration of each. Selecting and balancing counter-ions to active metals, as well as the buffers and acid/bases, is very important for some recipes. Lipids and very hydrophobic ingredients usually require their own separate concentrate solutions (27).

Common goals for feed supplements include providing generally depleted nutrients, adding a particular substrate to drive an alternative metabolic pathway, or introducing materials to specifically influence apoptosis. But another category alters culture metabolism from growth into product accumulation mode. Such feed ingredients

- change ambient pH (up or down) or overall tonicity (higher or even lower)
- increase a specific ion complement (e.g., acetate)
- specifically inhibit cell division or DNA replication (28, 29)
- introduce toxicants or cytostatic agents (such as butyrate).

**Apoptosis:** Bioprocessors often want to maintain high-density cultures for as long as possible, so apoptosis is a common problem. Approaches to controlling it include recombinant expression of such apoptosis suppressors as the protooncogene bcl-2. Small-scale benefits have been reported for supplementation of cultures with such directly antiapoptotic agents as the FAS-receptor-mediated caspase inhibitor Z-D-CH<sub>2</sub>-DCB (30). Another way is to supplement cultures at an appropriate time with identified nutritional components, antioxidants, or growth factors.

## PROCESS DEVELOPMENT

Optimization of a fed-batch process involves a delicate interplay between such variables as feed-solution constituents and concentrations, the timing and duration of feed introduction, and control limits for reactor operating parameters (31, 32). So feed-process characteristics are as useful as feed-solution composition information (33). First, case-specific operating and performance parameters are identified based on characteristics of the host cell-line and bioreactor. Then specific goals of a process are set, as detailed in the “Goals” box (34). The number of factors interacting in these systems make research expertise regarding particular cell lines and bioreactors, as well as recently introduced aids such as DOE principals, important to process development.

**Scaling Down:** Much feed/process optimization today is accomplished in scale-down physical models built by imitating conditions existing in full-scale production reactors (35). Often the best small-scale conditions are sacrificed in favor of approximating those that can be actually achieved in a large-scale system. Essential feed composition

**Table 2:** Feed components

Factor	Benefit	Example
Nutrient concentrates	Increase both biomass and product	10× MEM AA
Cell-line/clone-specific	Cell lines can have unique demands	Lipid dispersions
Production enhancers	Increase cell-specific productivity	Butyrate
Acid/base/buffers	Restore pH to cell growth optimum	1N NaOH
Acid/base/buffers	Adjust pH to productivity optimum	Shift 7.3 to 7.0
Antiapoptotics	Promote culture longevity/production	Antioxidants
Surfactants	Reduce shear stress and foaming	Silicone-based

### REASONS FOR POPULARITY OF FED-BATCH CELL CULTURE

Variant of simple batch mode, for which most cells are already well characterized

Quickly, inexpensively, and easily implemented relative to other systems

Reproducible/predictable of large-scale system modes (after batch)

Allows significant latitude in materials and in process design, adaptation, and optimization

Offers significant production levels: more than batch and in league with the best

Supports metered introduction of concentration-sensitive ingredients (e.g., glucose)

Accommodates many specific implementation/optimization approaches publicly available

Works well for secondary metabolites (includes non-growth-associated products such as recombinant proteins)

Allows alteration and control of both absolute and relative nutrients levels

Presents a small footprint, promotes efficient use of media, and produces less waste than perfusion

Generates good lot consistency

Eases process characterization and validation

Supports efficient raw harvest, downstream clarification, and product concentration

and timing information determined in 30-mL to 100-mL suspension shake-flask culture are often used as initial conditions in fully controllable small-scale bioreactors with operating volumes of 0.5 to 20 L.

Newer technologies promise to accelerate optimization with more accurate scaled-down conditions. For example, SimCell reactors from BioProcessors ([www.bioprocessors.com](http://www.bioprocessors.com)) are miniaturized bioreactors

that use microfluidics, permeable membranes, and advanced optical measurement systems to produce scalable results in 150- $\mu$ L to 1000- $\mu$ L reactions. Such apparatus have robotic devices that allow simultaneous design, management, and monitoring of thousands of cell culture experiments (36). Furthermore, developments in on-line and real-time monitoring of nutrient, product, and waste levels have contributed to the speed and efficiency with which feed composition and timing are developed (37).

**Reactor Modeling:** Small-scale bioprocessors can enjoy a boost in productivity from simple, generic, ad hoc feeding, but large-scale manufacturers in regulated environments must optimize and control their procedures. To do so, they first develop equations describing the elements of bioreactor operation (process conditions). Equations can be developed from two basic sources: theoretical mass/energy relationships of metabolic reactions or nutrient and product level values from actual bioreactor experiments. Equations are compiled from those sources to produce mathematical models used to simulate real systems (38).

Mathematical simulations of actual bioreactor runs suggest how variables such as substrate set-point concentrations, feeding time-step patterns, and concentration of feeding solutions should be “tuned” to elicit a desired response. Insights gained from modeling can guide in the adjustment of a process, eliminating unnecessary rounds of characterization. Finally, comparing actual experimental results with model predictions helps improve the models over time (39).

### MONITORING AND CONTROL

Most people in bioprocessing are

familiar with the basic monitoring instrumentation of small-scale commercial bioreactors that continuously reports such intrinsic culture values as dissolved oxygen (DO), pH, and temperature using electrochemical sensors. Beyond that, many culturists use off-line procedures that provide data on the state of cells and culture media (both substrates and products) as well as the physical environment of a reactor. One example is daily sampling that measures secreted product accumulation using such techniques as ELISA or HPLC.

A need for more and rapid monitoring techniques is being driven by the demand to build better mathematical models; a general desire to better control feeding and other operational variables; new requirements to produce repeatable, transportable, and operator-independent processes; and the need to comply with the FDA’s process analysis technologies (PAT) initiative. New off-line biochemical methods are becoming available as well as on-line, noninvasive, and quantitative tools and techniques to monitor new aspects of bioreactor systems. One system from YSI Inc. ([www.ysi.com](http://www.ysi.com)) allows real-time monitoring of 10 specified nutrients and metabolites.

Up-and-coming means of reactor monitoring include an interferometer-based sensor technology that supports automated, continuous biochemical analysis of a culture process. Such systems are based on a totally noninvasive, near-infrared optical instrument that provides simultaneous values for hundreds of culture components in real time. And new off-line, same-day monitoring techniques (e.g., for product glycoconjugates using lectin array-based techniques) show great potential (37).

**Control Systems:** Many advanced control algorithms are now available — and are constantly being improved. Bioreactor control methods compare either measured or derived values with mathematical simulations to determine the timing and interval of feed introduction (40). Such orchestrated nutrient supplementation is implemented in a variety of basic approaches such as

open- or closed-loop control models. Standard “closed-loop” methods are based on precise mathematical formulas. “Open loop” methods use real-time process measurements that “feed back” to controlling devices and guide their actions (41, 42).

Sometimes a simple formula (however advanced) cannot describe a process, whether because of a lack of accurate real-time data, the nonlinearity of many functions, or delays in biological response to control inputs. In newer approaches, statistical and nonformulaic techniques such as “fuzzy logic” and “neural networks” are becoming prevalent (43). (*Editor’s Note: See this month’s Vendor Voice by Meg Kay for more information.*) Thankfully, cytotechnologists and reactor operators need not understand the mathematical basis of such approaches — or even details of their algorithms — to apply them efficiently as part of a control scheme.

Process control algorithms are integrated into electronic and mechanical hardware to provide reactor control. A proportional-integral-derivative (PID) type of controller is standard in most industrial control applications. One important service such controllers can provide is to assist in bringing values to their set points and maintain them without overshooting.

First a cell line’s behavior in a particular medium, bioreactor, and operating procedure must be understood. Then a mathematical model can be optimized and a local controller (e.g., PID) programmed to accept set points for measured reactor variables (e.g., from an on-line YSI analyzer) to control various actuators (e.g., peristaltic pumps) for maintaining predetermined set points (e.g., glucose levels) and a generally higher level of process control.

## LOOKING TO THE FUTURE

Many commercially important biologicals are produced by mammalian cells, and the fed-batch approach is the most popular means of

their culture. Advances in the understanding of high-density serum-free animal cell culture have provided a basis for high-yielding bioreactor production. Improved bioreactor engineering, mathematical modeling, and bioreactor control have increased efficiencies in large-scale fed-batch approaches, resulting in dramatically increased peak cell densities and net production.

Today’s experienced fed-batch bioreactor operators can expect up to 10 times the efficiencies possible just a few years ago: Yield for some secreted recombinant protein biologicals is now expected to be 1–3 g/L of culture, with 5–10 g/L potentially achievable. As the newest research is applied to production scale, yields exceeding 10g/L are anticipated.

## ACKNOWLEDGMENTS

I would like to thank Christian Julien (an independent consultant, christian.julien@rcn.com) for his suggestions and comments in the preparation of this manuscript.

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## GOALS IN FEED TYPE AND TIMING

- Replenish consumed substrates/metabolites (e.g., glucose, AA).
- Support cell-line, clone, or selection system requirements.
- Promote high product accumulation over mere culture mass.
- Promote high product quality (e.g., glycosylation) over quantity.
- Support product and processing pathway-specific metabolisms.
- Consider the operational capabilities of the bioreactor/services.
- Maintain desired metabolic pathways at high cell densities.
- Avoid toxicity or undesired metabolism at each stage of culture.
- Prolong cell viability and inhibit apoptosis at high cell densities.
- Induce product accumulation over cell division at latter stages.

## FEED CONSTITUENT USES AND CHARACTERISTICS

**Acid/Base/Buffer:** pH adjustment (many have concentration limits)

**Amino Acids:** individual cell line, clone, and product specificity

**Anti-Apoptotics:** FAS-receptor mediated caspase inhibitors, for example, protect against apoptosis.

**Antioxidants:** substrate specificity on both nutrients and cells

**Cell-cycle Actives:** for secondary metabolite accumulation over cell-mass

**Expression Actives:** often timing, concentration, and cell-line specific

**Glucose:** High levels promote toxic lactate production.

**Glutamine:** High levels promote toxic ammonia production.

**Hydrolysates:** Although not chemically defined, plant seed digests remain popular.

**Iron Transporters:** cell and media specificities, precipitation concerns

**Lipids:** Cell-specific requirements and precipitation concerns.

**Metals:** Availability of some are reduced during culture.

**Nucleosides:** metabolite function, selection system specificity

**Processing Actives:** support harvest stability, clarification, and concentration

**Surfactants:** Optimal activity can depend on addition timing.

**Vitamins:** Availability of some are reduced during culture.

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