

Supplementation of Animal Cell Culture Media

by William G. Whitford

Since Harry Eagle's development of synthetic animal cell culture medium in 1955, culturists have found the need to supplement, or add ingredients to, standard media formulations. Supplementation can occur at the time of media preparation from powder, as when adding sodium bicarbonate, or ingredients can be added to sterile liquid media, such as when adding animal serum to commercial liquid formulations. It can be a simple step, as in the addition of a sterile antibiotic solution at the time of culture, or a sophisticated process, as in model-determined multistep fed-batch culture. Determining what supplementation may be used in a particular application involves such things as examining the nature of the base (or basal) medium, the cell line used, and the application at hand, as well as considering what components may be tolerated by both the immediate culture and subsequent downstream processes.

Specific factors considered in culture supplementation include the time and amount of addition; the supplement's physical state (solution, emulsion, or powder) and components (from metal salts to high-molecular-weight protein); stability of components (both physical and chemical); stability and processability of the final (postsupplementation) medium; and the degree of process optimization required. Interestingly,



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most materials added as supplements, including such common ones as serum, can be included as part of standard formulations. There are a number of distinct reasons for their omission and subsequent specification as supplements: allowing end-users to specify their unique initial concentrations of each separate supplement; to change the concentration throughout an application; to produce unique combinations of such materials; and to select specific vendors for each separate ingredient.

BASAL MEDIUM

Major advances in culture media include serum-reduced and serum-free media (SFM). SFM have in turn evolved into protein-free (PF), animal-derived component-free (ADCF), and chemically defined (CD) formulations. Early, or classical,

media were designed to be supplemented with serum and to support a wide spectrum of cells and applications. Those media balanced amino acid and carbohydrate levels to culture densities achievable with serum supplementation combined with known types and levels of such cofactors as vitamins and trace metals. Common classical media include such familiar names as MEM and RPMI 1640 (see Chapter Two). They rely upon serum to supply much of their nonnutritive culture-promoting potential.

However, the standard medium for many users has become SFM, and each such formulation is often more specialized in its cell line and application potential than are the classical media. This trend is occurring at a time when more and more cell lines and unique culture applications are being developed. From the isolation of rare and specialized cells (such as mesenchymal stem cells) to ex vivo culture cell-based therapies (e.g., T cells) to the 20,000-L scale production of recombinant products (mainly monoclonal antibodies and other proteins), highly specialized applications demand more function and efficiency from the media used. Such developments have increased the frequency and diversity of culture media supplementation.

SUPPLEMENTS

Hundreds of individual compounds can be added to cell culture media,

ABBREVIATIONS USED

ADCF: animal-derived component-free
bFGF: fibroblastic growth factor (basic) or FGF-1
BPE: bovine pituitary extract
CD: chemically defined
CHO: Chinese hamster ovary
DPM: dry powdered media
FBS: fetal bovine serum
FGF: fibroblastic growth factor
Geneticin: G-418 sulfate aminoglycoside
GM-CSF: granulocyte/macrophage colony-stimulating factor
HMW: high molecular weight
IGF-1: insulin-like growth factor one
IgG: immunoglobulin gamma
IL-1: interleukin one or lymphocyte activating factor
IVF: in vitro fertilization
LMW: low molecular weight
MEM: minimal essential medium
MSX: L-methionine sulfoximine
PF: protein free
PHA: phytohemagglutinin
PVP: polyvinylpyrrolidone
QC: quality control
RPMI 1640: Roswell Park Memorial Institute 1640 medium
SFM: serum-free media
TGF- β : transforming growth factor beta
Z-D-CH2-DBC: Z-Asp-(2,6-dichloro-benzoyl)oxymethane

especially in the interest of researching their specific effects. But the concept of media supplementation is usually limited to addition of materials that generally promote establishment and maintenance of a cell culture. These media supplements come in a wide range of molecular types and physical states. They vary in complexity (from a defined amino acid to partially hydrolyzed animal tissue), in chemical nature (from gentamicin to glutamine), and in molecular weight (from calcium chloride to methylcellulose). Furthermore, particular supplements can be obtained in a number of packaged states, such as frozen liquid or lyophilized powders. Along with the diverse number of media and applications in which any one can be used, the many variations prohibit specific formulation, filtration, dosage, and storage information from being supplied here. However, some general principles can be mentioned.

Factors to research in the application of any supplement include cell-culture-use validation of the source material; methods of intermediate processing such as rehydration, sterilization, aliquoting, storage, and testing; timing, means, and amount of supplementation; and ancillary effects of the supplement on other aspects of the culture or downstream processing. Supplement contraindications occur in the areas of regulatory compatibility (e.g., adding protein to a protein-free medium), process compatibility (e.g., some supplements cannot be filtered once diluted in SFM), and component compatibility (e.g., some combinations of antibiotics can be toxic in SFMs). Because even “ultra-pure” reagents can demonstrate toxicity or lack of performance in some cell cultures, it is important to select only those

validated for a given application. For practical reasons, liquid supplements other than serum must be concentrated in the range of 50–5000 \times the desired final concentration (1).

Animal blood serum is by far the most common supplement to culture medium. It provides necessary proteins, growth factors, hormones, lipids, vitamins, attachment factors, and trace metals for culturing cells in leaner classical media formulations. Because serum has been used in cell culture for so long (actually over a century), numerous “dogma” about material selection and use have arisen, and the value of such reports varies greatly. Examples include basic questions regarding acceptable storage conditions (such as frozen or refrigerated), filterability after dilution, and heat treatments. Objective and systematic assessments do exist, however, and they can help users in the choice of specific products and their most efficient uses.

Dozens of serum types are available to choose from, and basic factors include the source animal species; age of animals at the time of collection; geographic origins; the means of blood collection and processing; and a number of postproduction quality control (QC) and lot-designation considerations. For specific cell line and culture purposes, factors to be considered in serum choice include its culture attachment (some cells will grow in suspension; others must attach themselves to supports) and growth promotion potential, lot-to-lot reproducibility, efficiency in supporting product generation and purification, purity from both chemical and biological contaminants, and regulatory status.

An additional category of sera are those screened and found suitable for specialized applications, such as mammalian stem cell or insect cell culture. Recently, serum suppliers have provided so-called “fortified sera,” which for identified applications can offer better performance at a lower price. Such products are defined types of serum that have been fortified (supplemented) to provide additional or improved performance. The nature of the supplements added may or may not be available from a given manufacturer, but generally they include such low-molecular-weight ingredients as vitamins, minerals (e.g., iron), and amino acids. Some specially prepared sera also have been modified by such processes as adsorptive chromatography, heat treatment, and diafiltration to offer one or more particular characteristics such as reduced concentrations of IgG, lipids, or salts (2).

Serum is frozen shortly after processing and maintained in that state until the time of use. Culturists generally thaw the product at 37 °C with occasional mixing and aseptically add it at 1–20% v/v to otherwise full-complement media. Here are some errors to avoid at this stage:

Purchasing a Type of Serum for an Application Simply Because a Relevant Reference Cites It: Understanding what specific properties in individual serum products are important to each cell line and application has grown, and it is very possible that greater efficiency and economy can be obtained through the efforts of screening specific serum products.

Incubating Frozen and Thawed Serum Without Gentle Mixing: A gradient can be established upon stationary thawing, which can cause the most concentrated

Table 1: Culture media supplements

Supplement	Description	Purpose	Commercial Example
Sera	Cell-free, highly processed animal blood fraction	Natural source of growth factors, lipids, vitamins, attachment factors, metals	HyQ characterized FBS (HyClone, Logan, UT)
Fortified sera	Serum with added nutrients/co-factors	Provide more powerful and economical serum effects	HyQ bovine growth serum (HyClone)
Screened sera	Selected lots of sera tested for performance in a specific application or cell line	Provide assurance that a lot of serum will perform consistent with past experience	HyQ ES screened FBS (HyClone)
Serum fractions	Mixtures of selected serum components	Provide more of identified beneficial serum components	EX-CYTE (Celliance, Norcross, GA)
Serum replacements	Cocktails of protein, nutrient, vitamin and growth factors	Replace, extend, or supplement serum in classical media	Lipumin (PAA Labs, Les Mureaux, France)
Tissue extracts	Mixtures of selected plant or animal tissue components	Natural source of unique or added growth factors, and so on	BPE (Fisher Scientific, Pittsburgh, PA)
Hydrolysates/peptones	Partially acid hydrolyzed or enzymatically digested tissues/cells/seed flours	Natural source of unique or added peptides, amino acids, vitamins, carbohydrates	Yeast extract (Marcor, Carlstadt, NJ)
Hormones/growth factors	Cell-proliferation-active polypeptides, proteins, steroids, and so on	Provide for cell- or application-required pathway regulation and culture proliferation	IGF-1 (Biosource, Camarillo, CA)
Cell cycle regulators	Lectin-based mitogens and synthetic caspase inhibitors	Promote division and inhibit apoptosis in, e.g., primary culture	Z-D-CH2-DCB (Calbiochem, San Diego, CA)
Matrix/attachment factors	Cell attachment/association active gelatin, collagen, laminins, fibronectin, and so on	Provide for cell association/attachment to microcarriers, flasks, 3D substrates	ZHS 8949 (TCS CellWorks, Buckingham, UK)
Antibiotics/antimycotics	Metabolism-based inhibitors of bacteria and fungi	Prevent, control, or eliminate adventitious agents in culture	Pen-Strep Solution (HyClone)
Nutrient/vitamin concentrates	Defined cocktails of amino acids, vitamins, metal ions	Provide additional culture or production capacity in media	NEAA 100x (HyClone)
Simple nutrients/salts	Glutamine, glucose, pyruvate, Na ₂ HCO ₃ , CaCl ₂	Special cases that require subsequent addition	L-Glutamine (HyClone)
Lipid concentrates	Stabilized dispersions of sterols, fatty acids, lecithin and fat soluble vitamins	Provide final lipid product for auxotrophs and complex precursors for efficiency	HyQ LS250 (HyClone)
Selection agents	Toxins, analogs, inhibitors, salvage pathway precursors	Exogenous gene selection/amplification/maintenance	MSX (Sigma, St. Louis, MO)
Media kits	Sets of basal media, supplement mixtures, and defined supplements	Allows user modification of media for optimization to an application or cell line	ProMEDIA SELECT (Cambrex, Nottingham, UK)
pH buffers	Selected Good buffers, NaH ₂ PO ₄ , Na ₂ HCO ₃ , β-Glycerophosphate	Provide additional or novel buffering for specialized or enhanced culture/production	Hepes buffer 1M (HyClone)
Iron transporters	Iron associated with natural or synthetic chelators	Maintenance of intercellular iron in stressful applications	Human Transferrin (Celliance, Norcross, GA)
Synthetic HMW polymers	Methylcellulose, polyvinylpyrrolidone, pluronic acids	Provide altered viscosity, rheology, shear-protection	Methylcellulose (Fluka, Mulhouse, France)

components settled at the bottom of the container to precipitate out of solution.

Adding Serum to SFM: Many SFM are so optimized to serum-free applications that the addition of serum can actually inhibit their performance.

Several **serum fractions and extracts** provide a concentrated (and a bit more defined) source of certain active serum components. For example, lipid-rich serum fractions can be harvested and concentrated as a stabilized emulsion to be added as a media supplement at the time of use. They can provide better economy and control in cell lines that are responsive to those particular serum components.

The timing and method of both extraction and filtration have been found to be significant factors in determining the activity of a final extract. Because of that, and depending on what constituents of the serum are effective with a particular cell line or culture, the value of any particular commercial preparation should be determined in specific application.

Some materials, although referred to with a succinct name, are actually rather refined extracts or fractions. For example, bovine serum albumin (BSA) is a blood product very commonly added to otherwise complete SFM formulations, especially in such culture-stringent applications as

cloning assays. Although more defined than most “extracts,” it usually exists as a mixture of chemicals (including fatty acids and HMW proteins, for example) and thus can suffer from the same concerns regarding lot and production method variance as serum and cruder serum fractions.

Tissue extracts, such as bovine pituitary extract, provide media with an undefined number of hormones and growth factors. They are aqueous or polar solvent-derived cocktails of many culture proliferation-active components. As with serum and serum fractions, their performance in any particular application can vary greatly with the quality of the original animal

material, lot-determined component variation, and the means and expertise of preparation. As with BSA, other extracts (such as fetuin, lecithin, and even phosphatidylcholine) can have simple names implying a defined composition, but they are actually mixtures containing many (and even disparate) molecular constituents.

Serum “replacements” of many types and basic compositions have been commercially available for years. Similar to serum, they come as liquid concentrates to be added to media, but they can provide advantages in consistency and economy. Although these additives can provide a value in some media and applications, the fact that their compositions are often undefined — and that they might even include serum as a component — reduces their general utility in today’s SFM product and regulatory environment.

Hydrolysates and peptones are produced through the acid hydrolysis or enzymatic digestion of tissues (such as beef), cells (such as yeasts), or seed flours (from soy, for example). They are an old and well established means of adding inexpensive and natural sourced peptides, amino acids, vitamins, metals, and carbohydrates. Most of the earlier SFM relied heavily upon them, and they are still a component of many high-performance production formulations. For instance, yeastolate is still used as to provide extra power to SFM formulations and is even an absolute requirement of some (insect cell SFM, for example). However, these additives are falling out of favor in some circles because of potential consequences arising from the uncharacterized nature of such very complex mixtures — especially issues with inter-lot performance consistency (3).

Cytokines, hormones, and growth factors are required supplements for many cell culture applications, especially those using SFM. Their compositions can include such diverse molecules as steroids, aromatic amino acid derivatives, polypeptides, proteins, and glycoproteins. They are often required for such culture functions as cell differentiation, morphogenesis, proliferation, activation, and migration, but their activity is often specific to each basal medium, application, and cell line.

Of the dozens used in cell culture, some of the more common include basic fibroblast growth factor (bFGF), granulocyte/macrophage colony-stimulating factor (GM-CSF), transforming growth factor beta (TGF- β), interleukin-1 (IL-1), insulin-like growth factor (IGF-1), insulin, and hydrocortisone. Insulin, for example, is available as naturally sourced (e.g., from bovine pancreas extract) or recombinant (including many isomers and truncated forms of human insulin). Different insulins tend to have different biological activities as well as pH- and ion-dependent solubility, microaggregation, and folding behaviors. So dissolution, filtration, application, and storage in solution are issues in their use, and optimal procedures for handling vary with each form and source (4).

Cell-cycle and apoptosis-regulating agents of many types and applications include the long-used mitosis-inducing (mitogenetic) isolectins of phytohemagglutinin (PHA) and, recently, FAS-receptor-mediated apoptosis controlling caspase inhibitors such as Z-D-CH₂-DBC (the “Abbreviations” box lists those not defined in text). The activity of such agents includes their direct influence on cytokine and chemokine regulation.

Attachment factors and extracellular matrix (ECM) proteins are another heterogeneous group of molecules required in the SFM culture of some cells for particular applications. Agents active for cell attachment and/or adhesion include such materials as gelatins, collagens, laminin, fibronectin, vitronectin, fetuin, and other proteoglycans. Examples of ECM functions include integrin activation and cell-to-culture substrate interaction. Some of these materials can even be added to a medium to help cells attach to microcarrier beads or culture flasks (5).

Antibiotics and antimycotics became popular supplements when they were identified and used in pharmacology. In cell culture, many compounds have been found to prevent or even treat bacterial, mycoplasmal (PPLO), fungal, and even some viral contaminations. The most popular supplements in this regard are frozen solutions of

penicillin/streptomycin, gentamicin, and amphotericin B (Fungizone) and Mynox (from Minerva Biolabs GmbH, Berlin, Germany). Unintended effects of such supplementation can include maintenance of troublesome subvisible culture contamination and the activity (including toxicity) of the antibiotics on animal cell metabolism. Because of those risks — and the implementation of better aseptic techniques and equipment — many have restricted the use of these additives to specialized instances.

Concentrates of nutrients and vitamins are commonly used to either provide additional potency to existing liquid media or allow cultures to operate in what is termed the fed-batch mode. Fed-batch is a popular means of increasing peak cell densities or duration of culture — or to provide for the accumulation of higher yields of biological products than can be achieved in normal batch culture. In fed-batch mode, cultures are treated normally up to a predetermined stage(s), and then nutrient supplements are added, usually as liquid concentrates. Generic concentrates are commercially available, such as 100× MEM amino acids, but many culturists (especially large-scale bioreactor operators) prefer to make their own customized cocktails using many component types and concentrations.

Particular Standard Components: Although typical constituents of most media, simple nutrient supplements such as L-glutamine and glucose are often added as supplements because of shelf life concerns and commonly observed issues involving the generation of metabolic waste products. In some solutions L-glutamine can spontaneously decompose into ammonia and pyrrolidone carboxylic acid. Therefore, although it can be added as a powder at the time of liquid media preparation, it is often added as a liquid supplement from frozen concentrate at the time of use.

Both L-glutamine and glucose are required by most animal cell cultures, but with some cells high concentrations of these ingredients can lead to unacceptable levels of ammonia or lactic acid production. Therefore,

Photo 1: A dual bioreactor capable of midculture supplementation (determined by, for example, pH, dissolved oxygen, and glucose levels) using integrated pumps and time, weight, or level control (SARTORIUS BBI SYSTEMS, INC., WWW.BBRAUNBIOTECH.COM)



many advanced culturists prefer to use them sparingly in initial formulas and complement them with other metabolic cycle participants — or add them as supplements in metered doses throughout a culture.

Sodium bicarbonate is invariably included in most liquid formulations but almost always left out of dry powdered media (DPM) because it can liberate CO_2 in storage. Therefore, between 0.4 and 4.0 g/L is added as a powder at the time of liquid media preparation. One role of bicarbonate is to work with the CO_2 present in an incubator to control the pH of a culture. More advanced procedures call for media to vary from the customary dose at the time of formulation and (in conjunction with a varying ambient level of CO_2 in the incubator) allow the control of media pH in use.

Calcium concentration (usually as CaCl_2) in ambient media is a modulator of particular cell functions. For example, changing its level can contribute to determining a cell's propensity toward suspension or adherent culture. Therefore it has become popular to produce some specialty media products with a minimal amount of calcium and allow

for the appropriate amount of a 0.5-M concentrate to be added by users to bring the final concentration up to 20–400 mg/L at the time of use.

Lipid dispersion concentrates have become more popular of late in such activities as the SFM culture of lipid-dependent cells or in increasing the general performance of many cultures. Even when not essential, providing cells with appropriate preformed lipids such as fatty acids, phospholipids, and sterols reduces the need for their biosynthesis by the cell, which can lead to more efficient metabolism for industrial cultures. Because of the very limited solubility of lipids in water, significant engineering and dispersion-forming materials selection goes into devising acceptable formulations of sterile concentrates for cell culture. Considerations for them (beyond initial performance) include that upon dilution they should disperse to a biocompatible form and be taken up by the cells in a controlled fashion, that they can be microfiltered, and that they remain stable in storage for up to a year. Lipid concentrates should have their dilution filterability validated because most cannot be subjected to filtration once diluted in media (6).

Selection agents used in the generation and maintenance of recombinant cell lines are commonly added as supplements to bioindustrial media formulations after their initial preparation. These supplements consist of specialized metabolic pathway inhibitors, nutrient precursor analogs or agonists, and salvage pathway precursors. Not only do they support a selective pressure that allows only cells with an effectively incorporated gene(s) of interest to prevail in culture, but they can also provide a means of amplifying the recombinant product within the cells or supernatant. Common examples include G418 sulfate, L-methionine sulfoximine (MSX), and glutamic acid. One reason such agents are added as supplements is that individual culturists using specific types of media can thus choose from dozens of specialized selection systems and supplements. Also, the concentration of these additives often must be carefully optimized or varied through the process (7).

For adjusting pH, **buffers** such as HEPES, Tricine, Na_2HPO_4 , NaHCO_3 , β -glycerophosphate, and certain amino acids may be added to standard media. The purpose may be to support specialized procedures and culture modes, to compensate for a troublesome cell line, or to increase the performance of a culture in an optimized application. This can be to supplement or even entirely replace the traditional carbonate/ CO_2 buffering system.

Iron transporters such as transferrin, tropolone, and ferric ammonium citrate are sometimes added to increase a medium's capacity for high-density culture or applications that stress media potency, such as cloning assays. Iron is indispensable for some metabolic processes, including the production of deoxyribonucleotides, and iron starvation arrests cell proliferation. Although transferrin receptors are expressed on all dividing animal cells, many other complexed irons have been shown to take the place of transferrin. Which transporter will work in an application has been observed to be very cell-line and basal-medium specific.

Synthetic HMW polymers of many molecular species have significant

applications in cell culture. From methylcellulose in stem-cell colony-forming assays to polyvinyl pyrrolidone (PVP) for in-vitro fertilization (IVF) procedures to pluronic acids used as shear-force protectants, some polymers provide distinct benefits to cell culture applications. Such materials usually have especially specific, sometimes unusual protocols for their preparation and supplementation to media.

MEDIA KITS

Media kits (sets of components for supplementation into a basal medium at the time of use) are growing in popularity. The main driver for choosing this type of system is a desire to conveniently optimize a final formula to a particular derivative cell line, clone, or biotechnology process. Kits supply convenience as well as a bit of their designers' expertise in the parameters of a given medium's supplementation and optimization.

FEEDING CULTURES

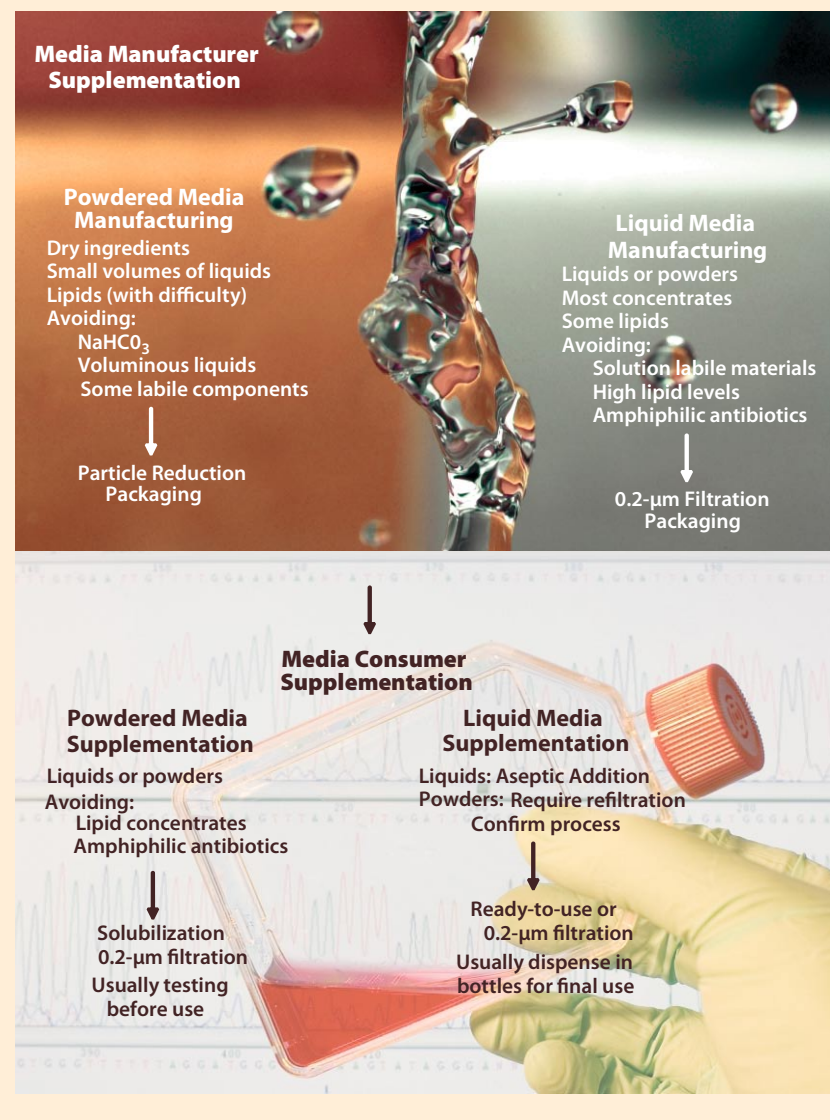
Supplementation for the purpose of culture feeding (as in fed-batch culture) deserves special attention here. Primary factors determining optimum feeding of cultures include

- preventing toxicity to initial low-density seed cultures
- maintaining desired metabolic pathways in high-density cultures
- prolonging cell viability and preventing induction of apoptosis
- promoting longevity of cultures over short-lived peak densities
- promoting high-quality product accumulation rather than mere quantity
- encouraging product accumulation rather than simple culture biomass.

The controlled supplementation of larger-scale systems has evolved into a discipline of its own. The goal of increasing harvested products can involve such means as increasing the number of cells (biomass) or increasing the secretion of product per cell. This usually requires supplementation of the culture media with certain gases, nutritional substrates, vitamins, growth factors, and/or specialty chemicals for altering cell metabolism.

The most common implementation involves the use of *bioreactors*, which are culture vessels allowing the control

Figure 1: Users and media manufacturers add different supplements




of such supplementation as well as many other elements of the total culture environment (Figure 1). The nonlinear and even discontinuous behavior of cultures in modes such as fed-batch and perfusion culture has led to the development of many engineering schemes or models. Orchestrated nutrient supplementation is implemented by variations of such schemes as segregated and unsegregated growth models and open- and closed-loop control models. Culturists planning for complicated or optimized supplementation procedures are referred to introductions of such systems (8).

SPECIAL CASES

Finally, by supplementation of media we usually refer to either the addition of new or increased levels of either powdered or liquid components at the

time of DPM dissolution before sterile filtration — or the aseptic addition of sterile liquid concentrates to packaged liquid media at the time of use. A number of less common special cases do exist and must be approached with an understanding of their potential for failure. They should thus be tested for functionality. For example, when adding nonsterile powders or lipids directly to finished liquid media, the effects of subsequent sterile filtration must be considered. When adding new ingredients to a medium or increasing the levels of those already present, culturists should not only anticipate the intended specific culture effects; they should also consider that such supplementation has the potential of altering a formulation's filterability, shelf life, toxicity, or one of many ancillary culture performance characteristics.

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