# Cell Culture Media for Recombinant Protein Expression in Chinese Hamster Ovary (CHO) Cells: History, Key Components, and Optimization Strategies

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*The culture of Chinese Hamster Ovary (CHO) cells for modern industrial applications, such as expression of recombinant proteins, requires media that support growth and production. Such media must support high viable cell densities while also stimulating the synthesis and extracellular transport of biologic products. Early media development efforts in this area yielded basic formulations to sustain growth, viability, and cellular function, albeit comprising animal sourced components, and complex constituents used in batch culture mode. Subsequent improvements included the development of serum-free and chemically defined (CD) media, the identification of critical nutrients, growth factors, and potentially inhibitory or toxic cellular metabolites, and the use of fed-batch and perfusion culture techniques to optimize nutrient delivery while minimizing accumulation of unwanted waste products. This review is comprised of sections covering milestones in the evolution of mammalian cell culture media, nutrient composition and formulation requirements, optimization strategies, consistency and scalability of powder and liquid media preparation for industrial applications, and key recent advances driving progress in CHO cell culture medium design and development.* © 2018 American Institute of Chemical Engineers *Biotechnol. Prog.,* 2018
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## Historical Evolution of Media Formulations to Support Mammalian Cell Culture

The earliest culture media successfully developed and used for the cultivation of animal cells were typically composed of plasma, serum, or tissue extracts.[1] The complex, undefined nature of these biological constituents resulted in variability, increased risk of contamination, and prevented the elucidation of the minimum, specific nutrient components required to support the growth of cells in the medium.[1,2] For these reasons, many research groups sought to develop serum-free, chemically defined cell culture media through analysis of cellular and serum composition, and identification of the key constituents needed to support growth.

Pioneering work in the laboratory of Harry Eagle,[3,4] led to a basic formulation, which could support the growth of two different cell lines, mouse-L fibroblasts and HeLa cells (human uterine carcinoma). Eagle's initial medium contained "an arbitrary mixture of amino acids, vitamins, cofactors, carbohydrates, and salts" along with dialyzed serum.[3] Based on analyses of the protein composition and amino acid metabolism of cells, Eagle further developed this medium into "Eagle's Minimal Essential Medium" (EMEM).[4] EMEM is comprised of 28 essential metabolites which, when supplemented with serum, can support propagation of a wide variety of cell lines with doubling times of 18 to 24 h.[4] Although this medium contains serum, its formulation is still the basis for many widely used cell culture media today.[5] For example, Dulbecco and Freeman's DMEM[6] was a modified version of Eagle's medium containing a fourfold concentration of amino acids and vitamins relative to EMEM. This formulation was shown to support enhanced growth and higher cell densities relative to EMEM, and was eventually used as the basis for a number of subsequent serum-free media formulations.[2]

Serum is a highly complex biological fluid containing a large number of nutrients, hormones, growth factors, carrier proteins, and metabolites, which support cellular growth.[7] The eventual development of serum-free cell culture media was driven by several factors, including undesirable characteristics of serum such as variability, risk of contamination with adventitious agents including BSE and TSE (Bovine and Transmissible Spongiform Encephalopathy), limited availability, and cost.[2] Serum is now generally considered undesirable for use in an industrial cell culture application from both process and regulatory standpoints.

Ham's nutrient mixture F12[8] was an early successful example of a fully synthetic, chemically defined, and serum-free medium which could support clonal growth of Chinese hamster ovary (CHO) cells. This medium was designed for single cell cultivation and expansion, but was not well suited to support growth to high cell densities (greater than $10^5$ cells per

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ml). Sato and colleagues later determined that this deficiency could be remedied by mixture of Ham's F12 with Dulbecco's medium, plus supplementation with hormones, growth factors, and transferrin, creating a new formulation called DMEM/F12.[9] Building upon this work, Hiroki Murakami and colleagues identified four critical additives necessary for the replacement of serum in chemically defined media: insulin, transferrin, ethanolamine, and selenium. Combined together, these four additives formed a supplement (ITES), which was used in place of serum in early serum-free media.[10] Furthermore, Murakami's team further improved upon existing media by mixing existing formulations, such as DMEM/F12 with others such as RPMI1640, an early cell culture medium developed at the Roswell Park Memorial institute (Buffalo, NY) for the successful culture of human lymphocytes.[11] Mixture of these two formulations, along with the ITES supplement to remove serum, generated the very effective and widely useful basal media RDF and, subsequently, enriched RDF (eRDF).[2,5,12–14] Yap and colleagues further evaluated the role of trace metals in early serum-free cell culture media, identifying insulin-mimetic activities of zinc, and demonstrating its use as an insulin replacement in chemically defined, protein-free media.[15,16] Table 1 shows the composition of several key historic media formulations described in this review.

Since these early achievements, the evolution of serum-free cell culture media has continued through development and optimization efforts aimed at supporting higher cell densities, viability and recombinant protein titer. Research groups at the Massachusetts Institute of Technology and the University of Minnesota pioneered the use of stoichiometric analysis of cell growth and nutrient utilization to design basal and feed media formulations to feed according to nutrient demand, thereby preventing depletion of key nutrients while minimizing accumulation of toxic metabolic waste.[17–21] Further optimization of CHO cell culture media and process parameters by leading teams in the biopharmaceutical industry, specifically aimed at commercial manufacturing of mAbs and other recombinant proteins, has resulted in dramatic increases in cell density and protein expression, with titers reaching more than 10 g/L in some cases.[22–32] Furthermore, several companies specializing in cell culture media have developed and optimized basal and feed media combinations specifically for recombinant CHO manufacturing processes (ThermoFisher, Waltham, MA, USA, GE Healthcare, Waukesha, WI, USA, MilliporeSigma, St. Louis, MO, USA, Lonza, Basel, Switzerland, Irvine Scientific, Santa Ana, CA, USA). The formulations of these commercially available media are typically proprietary, and although very limited data are available on cell growth, protein expression, metabolite profiles, nutrient composition, and utilization for these media, some researchers have compared and benchmarked these products.[33] Many of these vendors also offer media development and optimization services. Furthermore, SAFC (now MilliporeSigma) offers a very extensive and searchable knowledge base ("Media Expert") on its website, containing detailed information on all cell culture media components.[34]

## Key Components of Mammalian Cell Culture Media

All cell culture media require similar basic nutrients, which are essential to support life and cellular growth. Water, along with sources of Carbon, Nitrogen, and Phosphate, certain amino acids, fatty acids, vitamins, trace elements, and salts are all supplied in concentrations based on the chemical makeup of the cell, the calculated amounts required to reach a desired cell density, and knowledge of nutrient depletion rates so that critical components may be replenished to maintain and extend cell viability. Media has been developed for many different cell types, including microbial, plant, insect and mammalian, and with many different objectives in mind. While these diverse media may be generally similar in terms of the fundamental nutrient composition, this section primarily focuses on *serum-free*, chemically defined media developed specifically for industrial cultivation of CHO cells, and optimized for the expression of recombinant proteins.

### *Water*

Mammalian cells can be highly sensitive to impurities in water, which may include trace elements, bacteria, endotoxin (derived from bacterial cell membranes), trace organics and particulates.[2,7] To prevent contamination of the cell culture with these impurities, or inconsistent cell culture performance due to uncontrolled ion and trace element concentration in the media, highly purified water is recommended for use in cell culture media formulations. Typical water purification systems used to generate contaminant-free water include distillation/deionization, microfiltration, and reverse osmosis. Water purity is typically measured in terms of conductivity or resistance (>18 MΩ cm) as well as low organic carbon content (≤15 ppb), and should be free of endotoxin.[2,35] In many cases, Water For Injection (WFI) is used, as it is readily available, highly purified, and endotoxin-free. Many commercially available bottled water products designed specifically for cell culture meet these specifications, as well as being guaranteed free of mycoplasma, which can contaminate mammalian cell culture.

### *Energy sources (carbohydrates, glutamine, glutamate)*

In most media formulations, especially chemically defined media for CHO cell culture, glucose functions as the primary energy and carbon source. CHO cells transform glucose into pyruvate via glycolysis, and further oxidize pyruvate in the Krebs cycle to generate energy equivalent to up to 36 mol ATP per mol glucose.[36] Due to the relatively high activity and efficiency of the enzymes involved in glucose metabolic flux, especially in serum-free media,[37] CHO cells can maintain high viability in glucose-limiting media with glucose concentration lower than 3 mM (0.540 g/L).[38] It has been demonstrated that cell specific growth rate, cellular ATP concentration and amino acid metabolic rates do not decrease in a statistically significant manner along with decreasing glucose concentrations in the medium, as long as glucose concentration is maintained above 1.22 mM (0.220 g/L),[39] suggesting that glucose is not rate-limiting above this concentration. However, due to the rapid growth and nutrient consumption rates of CHO cells in mAb production media and process parameters, the glucose level in the medium is typically controlled at a much higher level, even in some glucose-limiting culture strategies.[40]

While glucose is the primary carbon source in typical CHO cell culture media, consumption of glucose usually leads to pyruvate accumulation and an increase in lactate concentration. High concentrations of lactate can be inhibitory to cell growth in mammalian cell culture.[41,42] Wilkens et al. observed that in the early stages of cell culture, the rate of lactate generation is not significantly affected by glucose concentration in the

**Table 1. Compositions of Key Historical Cell Culture Media**

| | Components (mg/L) | BME (Eagle 1955) | MEM (Minimum Essential Media) (Eagle, 1959) | F12 (Ham, 1965) | DMEM/F12 (Barnes, 1979) | eRDF (Murakami, 1989) |
|---|---|---|---|---|---|---|
| Amino Acid | Glycine | | | 7.51 | 3.76 | 42.8 |
| | L-Alanine | | | 8.91 | 4.46 | 6.68 |
| | L-Arginine | 17.42 | 105 | | 210 | |
| | L-Arginine HCl | | | 210.7 | 105.4 | 581.5 |
| | L-Asparagine | | | 13.21 | 6.61 | |
| | L-Asparagine Monohydrate | | | | | 94.5 |
| | L-Aspartic acid | | | 13.31 | 6.66 | 39.9 |
| | L-Cystine | 12.015 | 24 | | 48 | |
| | L-Cysteine HCl | | | 31.52 | 15.76 | |
| | L-Cysteine HCl $H_2O$ | | | | | 105.4 |
| | L-Glutamic Acid | | | 14.71 | 7.36 | 39.7 |
| | L-Glutamine | 292.2 | 292 | 146.1 | 657 | 1000 |
| | L-Histidine HCl $H_2O$ | | | | | 75.4 |
| | L-Histidine HCl | | | 19.16 | 9.58 | |
| | L-Histidine | 7.76 | 31 | | 62.0 | |
| | L-Hydroxyproline | | | | | 31.5 |
| | L-Isoleucine | 26.24 | 52 | 3.94 | 105.97 | 157.4 |
| | L-Leucine | 26.24 | 52 | 13.12 | 110.56 | 165.3 |
| | L-Lysine HCl | | | 36.52 | 18.26 | 197.2 |
| | L-Lysine | 29.24 | 58 | | 116 | |
| | L-Methionine | 7.46 | 15 | 4.48 | 32.24 | 49.2 |
| | L-Phenylalanine | 16.52 | 32 | 4.96 | 66.48 | 74.3 |
| | L-Proline | | | 34.53 | 17.27 | 55.2 |
| | L-Serine | | | 10.51 | 5.26 | 85.1 |
| | L-Threonine | 23.82 | 48 | 11.91 | 101.96 | 110.8 |
| | L-Tryptophan | 4.084 | 10 | 2.042 | 21.02 | 18.4 |
| | L-Tyrosine | 18.12 | 36 | 5.44 | 74.72 | 87.0 |
| | L-Valine | 23.44 | 46 | 11.72 | 97.86 | 109.0 |
| Salt | Calcium Chloride Dihydrate | | | 44.10 | 22.05 | 108.77 |
| | Calcium Chloride | 111 | 200 (0 for suspension culture) | | | |
| | Magnesium Chloride | 47.6 | | | | |
| | Magnesium Chloride Hexahydrate | | 200 | 121.98 | 161 | |
| | Magnesium Sulfate, Anhydrous | | | | | 66.22 |
| | Potassium Chloride | 373 | 400 | 223.8 | 311.9 | 373 |
| | Sodium Bicarbonate | 1680 | 2000 | 1176 | 2788 | 1050 |
| | Sodium Chloride | 5840 | 6800 | 7592 | 7196 | 6424 |
| | Sodium Phosphate Monobasic Dihydrate | | 150 (1500 for suspension culture) | | 750 | |
| | Sodium Phosphate Monobasic Monohydrate | 138 | | | | |
| | Sodium Phosphate Dibasic heptahydrate | | | 268.1 | 134.1 | |
| | Sodium Phosphate Dibasic Dodecahydrate | | | | | 6593 |
| Trace Elements | Cupric Sulfate Pentahydrate | | | 0.002497 | 0.001249 | 0.000749 |
| | Sodium Selenite | | | | 0.001729 | |
| | Ferrous Sulfate Heptahydrate | | | 0.834 | 0.417 | 0.222 |
| | Zinc Sulfate Heptahydrate | | | 0.8625 | 0.432 | 0.23 |
| Vitamin | Biotin | 0.2443 | | 0.007329 | 0.003665 | 0.1 |
| | Choline Chloride | | | 13.96 | 6.98 | 12.29 |
| | Choline | 0.1042 | 1 | | 2 | |
| | Pantothenate | 0.2190 | 1 | | 2 | |
| | D-Calcium Pantothenate | | | 0.4765 | 0.24 | 1.24 |
| | Folic Acid | 0.4414 | 1 | 1.3242 | 2.66 | 1.81 |
| | Niacinamide | 0.1221 | 1 | 0.03663 | 2.02 | 1.47 |
| | p-Aminobenzoic Acid | | | | | 0.51 |
| | Pyridoxal HCl | | | | | 1 |
| | Pyridoxal | 0.1672 | 1 | | 2 | |
| | Pyridoxine HCl | | | 0.06169 | 0.03085 | 0.51 |

***(Continues)***

**Table 1. Continued**

| | Components (mg/L) | BME (Eagle 1955) | MEM (Minimum Essential Media) (Eagle, 1959) | F12 (Ham, 1965) | DMEM/F12 (Barnes, 1979) | eRDF (Murakami, 1989) |
|---|---|---|---|---|---|---|
| | Riboflavin | | 0.1 | | 0.22 | 0.21 |
| | Thiamine | 0.2654 | 1 | | 2 | |
| | Thiamine HCl | | | 0.3373 | 0.1687 | 1.59 |
| | Vitamin B12 | | | 1.3554 | 0.68 | 0.34 |
| Polyamine | Putrescine 2HCl | | | 0.1611 | 0.08 | 0.040 |
| Growth | DL-α-Lipoic Acid | | | 0.2063 | 0.103 | 0.0516 |
| Factor | i-Inositol | | 2 | 18.02 | 13.01 | 46.85 |
| Fatty Acid | Linoleic Acid | | | 0.08412 | 0.04206 | 0.02103 |
| Other | HEPE | | | | 3574.5 | 1190 |
| | D-(+)-Glucose | 901 | 1000 | 1802 | 1401 | 3423 |
| | Hypoxanthine | | | 4.083 | 2.04 | 1.02 |
| | L-Glutathione, Reduced | | | | | 0.49 |
| | Phenol Red | | | | | 5 |
| | Sodium Pyruvate | | | 110 | 55 | 110 |
| | Thymidine | | | 0.7266 | 0.3633 | 5.72 |
| Additives | | 5-10% of serum, plus antibotics | 5-10% of serum | 5-10% of serum plus Phenol Red | Serum and antibiotics | serum-free |

medium, whereas in later stages high glucose concentration can result in increased lactate accumulation.[43] Analysis of metabolic flux has shown that in exponential phase, CHO cells largely generate energy via aerobic glycolysis and produce lactate regardless of the concentration of oxygen, while cells in stationary phase mostly perform oxidative phosphorylation and consume lactate.[44] These observations have led to the development of strategies utilizing low or limited glucose concentration in the culture media.[45,46] Meanwhile, to further reduce the effect of metabolites arising from glucose hydrolysis, a number of other energy sources have been evaluated as substitutions for glucose in the media.

Galactose is an important alternative carbon source, which may be used in CHO cell culture media. Like glucose, galactose is converted to pyruvate in glycolysis. Analysis of galactose metabolic flux has shown that in media containing both glucose and galactose, CHO cells tend to use glucose first followed by a shift to galactose consumption. This metabolic shift can lead to lower lactate production and higher lactate consumption, thus delaying a drop in cell viability.[43] However, complete replacement of glucose with galactose in the medium leads to reduced cell growth and an early drop in viability, despite well-controlled lactate and ammonium concentrations.[47] This is possibly due to the low activity of hexokinase for galactose. Presence of Galactose in the medium can also affect the expression of genes related to glycosylation. Gramer et al. reported that feeding galactose, along with uridine and manganese chloride could be used to control antibody galactosylation levels in a CHO cell culture process by adjustment of the concentrations of these three components.[48] Galactose feeding may also affect antibody sialylation by two different mechanisms. As galactose residues are the targets for sialyation in antibody N-glycans, increased galactosylation may therefore increase the potential sialylation level by providing additional sialylation sites. However, sialidase gene expression profiling and intracellular enzyme activity studies have suggested that galactose feeding may increase desialylation.[49]

Other hexoses have also been used as substitutes for glucose in CHO cell culture media,[50] including fructose, mannose, and others. Addition of mannose to glucose-containing media was shown to produce either no effect, or slightly reduced site-occupancy in N-Glycosylation.[51] Replacement of glucose with mannose in the medium may increase volumetric productivity by accelerating cell growth and reducing lactate accumulation without altering product quality.[52] However, media containing a high concentration of mannose can also inhibit intracellular α-mannosidase, thereby increasing the percentage of mannose glycosylation in the product, which can increase both antibody-dependent cell-mediated cytotoxicity (ADCC) and clearance rates of antibody in the human body.[53] Like galactose and mannose, fructose may also be used to control lactate accumulation and delay a drop in viability. However, it has been shown that media in which glucose has been completely replaced with fructose led to reduced cell growth and titer.[47]

In addition to glucose and other hexose sugars, glutamine is also utilized as a major energy source in CHO cell culture. Glutamine is metabolized via glutaminolysis and enters the Krebs cycle in the form of α-ketoglutarate. Glutamine is an essential nutrient in CHO cell culture, and its absence tends to delay the start of the exponential growth phase.[54] For CHO cell lines derived from glutamine synthetase gene knockout hosts (CHO-GS), media without glutamine is used to select for transfected cells.[55] However, supplementation of glutamine in basal media has been reported to improve cell viability, reduce lactate production and increase antibody productivity, even in the case of CHO-GS cells which produce abundant glutamine synthetase after transfection.[56,57] During glutaminolysis, however, glutamine is typically converted to glutamate, thereby also generating free ammonium in the medium.[58] High ammonium concentration in the medium can have a significant effect on the cell culture by potentially reducing cell growth rate, increasing the glycoform heterogeneity, and affecting the consumption rate of other amino acids.[59,60] Therefore, some approaches to medium optimization have been designed to either replace glutamine or reduce glutamine concentration. One such approach substitutes glutamine in the media with glutamine-based dipeptides, including alanyl-glutamine and glycyl-glutamine, in order to reduce the rate of glutamine metabolism and control ammonium release.[61] Currently, this technology is commercially available under the name Glutamax (ThermoFisher) and is commonly used in CHO cell culture.[62]

To reduce the accumulation of ammonium resulting from glutamine metabolism, glutamate, which is the primary intermediate of glutaminolysis, may be used as an alternative energy source in CHO cell culture media. Altamirano et al. reported that the replacement of glutamine with the same concentration of glutamate in a CHO cell culture medium can lower ammonium and lactate production rates in the production phase, thereby increasing cell growth and titer.[47] Furthermore, optimization of glucose concentration in a glutamate-based defined medium has been shown to result in higher efficiency of glucose and nitrogen source utilization, along with lower alanine and lactate production rates.[63]

Sodium pyruvate is another energy source, which may be used in place of glutamine in CHO cell culture media to reduce ammonium accumulation. Research has shown that the replacement of glutamine with pyruvate in CHO cell culture media does not have a significant effect on cell growth for at least 19 passages, and does not require any adaptation step to maintain the cell growth rate.[64] As pyruvate is an intermediate in lactate generation, and also the product of alanine dehydrogenation, pyruvate feeding can limit the consumption of alanine in the event of lactate depletion during CHO cell culture, thus reducing the generation of ammonium from alanine metabolism.[65]

### *Amino acids*

Amino acids are key components in CHO cell culture media, especially in chemically defined media. Studies have shown that small changes in amino acid composition of cell culture media can alter growth profiles and titers, and can also significantly affect product glycosylation patterns.[66] Optimization of amino acid concentration in culture media using metabolic flux analysis was shown to increase peak cell density by greater than 50% and titer by greater than 25% in a fusion protein production process.[24] In a more comprehensive study, Torkashvand et al. developed a workflow utilizing several Plackett-Burman experiments to optimize amino acid concentration in a cell culture medium by identifying key amino acids influencing cell growth and titers, and analyzing binary effects of those amino acids with response surface methodology. Using this approach, titer was increased by 70% without altering product quality.[67] In addition to enhancing titer and peak cell density, selected amino acids at certain concentrations may have protective effects for cells growing in bioreactors. Certain amino acids have been shown to eliminate or alleviate some of the negative effects of ammonium and $pCO_2$ accumulation, as well as high osmolality.[59,68] Some early reports suggested that some amino acids also act as signal molecules, reducing the rate of apoptosis in mammalian cells.[69] Therefore, amino acid concentrations should be carefully determined during medium design.

Generally, amino acids may be classified into nonessential amino acids (NEAA), which can be synthesized by mammalian cells, and essential amino acids (EAA), which cells are unable to synthesize and must therefore be supplied as components of the cell culture medium.[70] Response surface analysis of amino acids at multiple concentrations has shown both NEAA and EAA to have statistically significant effects on CHO cell growth. Furthermore, optimization of the relative concentrations of NEAA and EAA in the media formulation has been shown to improve the productivity of a recombinant monoclonal antibody.[71]

Essential amino acids include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. In most instances, all of the essential amino acids are required in cell culture media, and many of these have been shown to be consumed at relatively high rates in CHO cell culture processes.[72] Therefore, the essential amino acids are usually supplied at high concentrations in CHO cell culture media.

Lack of leucine, isoleucine, valine, or phenylalanine can increase the activity of transport system L by 1–4 fold, and increase the uptake rate of transport system L-related amino acids,[73] which will subsequently deplete those amino acids at a faster rate. Therefore, leucine, isoleucine, valine, and phenylalanine should be supplied at higher concentrations than those calculated by metabolic flux or "omics" analyses, and should be carefully monitored during cell culture.

Tryptophan has been found to be a limiting factor in some media studies. Tryptophan supplementation has been shown to increase both titer and peak cell density.[24] However, in a medium solution, tryptophan can be oxidized and transformed by fluorescent light into tetrahydropentoxyline, 5-hydroxy tryptophan, N-formyl kynurenine or other oxidation products, which may result in reduced cell growth.[74,75] Certain media formulation changes, including increased tryptophan, copper, and manganese concentrations, and decreased cysteine concentration, can reduce the photo-induced oxidation of tryptophan.[76] Nonetheless, protection from strong light exposure during media storage and handling is still the best approach to prevent tryptophan degradation and oxidization.

The inclusion of threonine in media can protect cells from various stresses in cell culture. Threonine is one of several amino acids, which has been shown to improve cell growth and productivity in environments with high ammonium, dissolved carbon dioxide or osmolality. By this mechanism, threonine and other amino acids can indirectly affect the sialyation pattern of the recombinant product.[59]

High concentrations of lysine and histidine in culture media can effectively reduce the acidic species of the final product without affecting productivity. High concentrations of lysine and histidine have also been shown to slightly reduce cellular growth rate in the exponential phase while improving cell viability in the late stage of a production process.[77] However, high concentration of lysine may have an inhibitory effect on basic carboxypeptidases, resulting in an increase in C-terminal lysine variants.[78] Therefore, the concentration of lysine and histidine may be changed based on specific product requirements.

The nonessential amino acids include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine. Despite the fact that the nonessential amino acids can be synthesized by mammalian cells in culture, most cell culture media still contain most or all of these amino acids to support cell growth and protein production. In fact, most of the nonessential amino acids have a significant effect on cell culture processes.

The initial concentration of alanine is relatively low in most media. Media studies have shown that cells tend to either consume alanine at a very slow rate or produce alanine from other amino acids during cell culture.[24,72] With sufficient lactate present in the medium, the alanine production rate is positively correlated to the amino acid concentration.[79] The accumulation of alanine has little direct inhibition on cell growth, unless the concentration of alanine exceeds 3 mM.[80] However, under conditions of lactate and pyruvate starvation, alanine tends to be converted to pyruvate and release free ammonium

into medium via glutamate degradation, thus increasing the concentration of ammonium in the culture.[65]

Arginine is consumed at a relatively high rate by CHO cells in antibody production processes.[81] Arginine starvation can cause CHO cells in exponential phase to be captured in the G0 or G1 phase.[82] Excess arginine in the culture medium can effectively reduce the ratio of the acidic species of products. However, unlike lysine, high arginine concentrations can inhibit cell growth and reduce viability.[77] Additionally, a rise in arginine concentration has been shown to double the amount of C-terminal lysine variant and thereby increase the heterogeneity of an antibody product.[83]

Asparagine and aspartic acid are important nonessential amino acids in cell culture media, playing a central role in key metabolic pathways. CHO cells tend to consume asparagine and aspartate at relatively high rates compared to other amino acids, especially during exponential growth phase.[84] Like glucose, glutamine, and glutamate, these two amino acids are important for the replenishment of TCA cycles and for energy metabolism. Aspartate and asparagine serve as ammonium donors and acceptors in the transition between glutamine and glutamate via the transaminase reaction, which can generate both glutamate and oxaloacetate.[85]

In the absence of asparagine, CHO cells may compensate with a higher conversion of aspartate, glutamate, serine, glutamine, and arginine, and may even consume the whole intracellular pool of amino acids at a higher rate; the resulting reduction in amino acid availability could then negatively affect protein synthesis or overall gene expression.[86] In fact, limitation of asparagine and aspartate in culture media has been shown to be detrimental for CHO-based monoclonal antibody production. Asparagine starvation during the growth phase, for example, is the major cause of the random misincorporation of serine at asparagine sites in the peptide sequence of antibody products.[87] Furthermore, asparagine plays an important role in the suppression of DNA damage and apoptosis.[88] Asparagine concentration in the media can also be utilized to control the distribution of galactosylated glycoforms of the product. Low asparagine concentration can reduce ammonium generation and intracellular pH, which subsequently enhances the activity and expression of β-1,4 galactotransferase, ultimately resulting in a shift from G0F to G1F/G2F.[89] Furthermore, increased asparagine concentrations in feed media can enhance the final titer at the expense of high lactate and ammonium. However, the ammonium concentration can be reduced by increasing the ratio of asparagine to glutamine in the feed medium.[90]

Cysteine, the only thiol-containing amino acid, is a special nonessential amino acid in monoclonal antibody production. The formation of disulfide bridges between sulfhydryl groups on cysteine residues supports the folding of tertiary and quaternary structure of both CHO cell structural proteins and recombinant antibody product. Cysteine limitation can be fatal and irreversible for CHO cell growth, and may lead to a cell viability drop below 40% in 3 days.[91] A drop in cysteine concentration below 0.1 mM in the production medium can result in a 40% loss of titer in monoclonal antibody production. Meanwhile, cysteine concentration of >1 mM can be toxic for mammalian cells,[92] possibly due to lipid peroxidation and formation of hydroxyl radicals, which can be further accelerated in the presence of copper.[93] In mammals, the cysteine pool is regulated by the liver.[94] Without such a regulating mechanism in CHO cells, cysteine concentration in the medium should be carefully designed and controlled for cell culture processes.

Glycine is consumed at a relatively slow rate by CHO cells, and is not a major component to be studied in most medium optimization efforts. In CHO cells, glycine is the by-product of de novo biosynthesis of thymidylate. However, for CHO DG44 cell lines, which are dihydrofolate reductase (DHFR)-deficient, the blocking of de novo biosynthesis of thymidylate can result in glycine starvation.[95,96] Therefore, the concentration of glycine in the medium for DG44-based CHO cell lines needs to be carefully designed, especially in expansion media which include methotrexate (MTX), a folate analog which blocks folate reduction.

Serine is the second highest consumed amino acid and the main donor for one-carbon (1C) unit metabolism in CHO cells.[97,98] More than 70% of the serine consumed by CHO cells in culture media is transformed to glycine, and ultimately contributes to thymidine synthesis.[98] Despite the important role of serine, low serine concentration in media (1 mM) seems to have little impact on cell growth, except for reduced formate and glycine production during the exponential phase. However, serine depletion in the medium can have negative impacts including increased asparagine consumption, alanine production, lactate production, and ammonium generation, which can ultimately inhibit cell growth.[99] Considering the high consumption rate and the adverse effects of depletion, serine is usually supplied at a relatively high concentration in the medium. In fact, some research has demonstrated that additional serine in the medium can improve titers and increase specific peak cell density.[100]

Tyrosine is a critical amino acid affecting productivity for mAb production in CHO cell culture. Low tyrosine concentration can interfere with protein translation and reduce specific productivity to nearly zero.[101] Tyrosine starvation is positively correlated to the extent of phenylalanine misincorporation at tyrosine residues, thus resulting in generation of product sequence variants. Maintaining tyrosine at a concentration above 1 mM in the culture medium can effectively eliminate misincorporation at tyrosine sites.[102] However, tyrosine is a poorly soluble amino acid, which complicates the use of high tyrosine concentrations in cell culture media.[103]

In summary, maintenance of most amino acids at specific concentration ranges in the media is very important for CHO cell culture. Therefore, feed media is usually designed to contain high amino acid concentrations. However, compared to other medium components, some amino acids have relatively low solubility and stability. To improve amino acid solubility, many feed media are designed at relatively high pH. For amino acid stability, media may be stored in the dark. Recently, some amino acid substitutions have been described with improved solubility and stability. For example, tyrosine, the least soluble amino acid, can be replaced with phosphotyrosine disodium salt or tyrosine-containing dipeptides to improve solubility.[103,104] Cysteine, one of the least stable amino acids, can be oxidized to cystine in neutral pH. The low solubility of cystine reduces the apparent solubility of cysteine in media. Recently, a highly soluble and stable cysteine derivative, S-Sulfocysteine, has been reported as a replacement cysteine source and anti-oxidant in CHO cell culture media.[105] Also, small peptides have been evaluated as alternatives to certain amino acids to provide improved stability and culture conditions.[106]

### *Lipids*

Lipids are major components of biological membranes, and can also serve as energy sources and signaling molecules in mammalian cells. They are key components of the endoplasmic

reticulum and Golgi apparatus, which are the organelles responsible for protein synthesis, folding, post-translational modification and secretion. Generally, CHO cells are able to synthesize lipids on their own. Recombinant CHO cells are capable of adaptation and growth in lipid-free media without a significant drop in cell proliferation rate and product activity. However, lipid supplementation in serum-free medium has proven beneficial for cell viability and product glycosylation.[107] In fact, the effect of lipids and lipid precursors on CHO cell growth can vary substantially depending on the types of lipids or precursors provided.[108] Therefore, the selection of lipid supplement used in the medium can have a significant impact on cell growth.

Phospholipids are major components of most mammalian cell membranes. Exogenous supplementation of phospholipids, such as phosphatidic acid and lysophosphatidic acid, has been demonstrated to stimulate CHO cell growth, regardless of the presence of growth factors in the serum-free medium.[109] As major constituents of phospholipids, choline and ethanolamine exhibit an enhancing effect on cell growth comparable to that of mixed lipid.[110] Choline supplementation has therefore been implemented in many well-known commercial media, including DMEM and RPMI-1640.

Fatty acids and cholesterol are not highly soluble or stable in culture media. As alternatives, different precursors and analogs of fatty acid and cholesterol, which are more stable and soluble in media, have proven useful in promoting cell growth and health.[111] Meanwhile, in the same study, a small amount of alcohol in the medium was confirmed to increase lipid solubility without influencing cell growth. Despite the importance of the involvement of fatty acid in media, the concentration of fatty acid should be kept at a relatively low level, as high concentrations of fatty acid may cause lipotoxicity in CHO cells.[112]

### *Vitamins*

Vitamins serve as coenzymes, prosthetic groups, or cofactors in signal cascade as well as in enzyme inhibition and activation. The high reductive capacity of vitamins can protect cells from oxidative radicals. Despite the trace amount needed, vitamins are essential components of cell culture media, especially in chemically defined media. Many commercial media contain B Vitamins and their derivatives, including biotin, folic acid, inositol, niacinamide, choline, 4-aminobenzoic acid, pantothenic acid, pyridoxine, riboflavin, thiamine, and cobalamin. Vitamin addition has been shown to increase mAb volumetric yield up to 3-fold in CHO cell culture.[113] However, not all vitamins typically included in media supplements are significant for cell growth.[114] For example, supplementation with folic acid, cobalamine, biotin, and 4-aminobenzoic acid was shown to be as effective in enhancing cell growth and productivity as increasing the concentrations of all vitamins in the medium.[100] Many vitamins are vulnerable to heat, strong light, and long-term air exposure. Particularly, among the vitamins commonly applied in CHO cell culture media, ascorbic acid and tocopherols are sensitive to air oxidation; thiamine, riboflavin, cobalamin and ascorbic acid are sensitive to light; and thiamine and pantothenic acid are sensitive to heat.[115] As a result, protection from light and heat are critical during storage of culture media.

### *Trace elements*

The effective concentrations of trace elements in cell culture media are typically very low, and in many cases, they can be below the detection threshold of standard analytical instrumentation. However, their importance is disproportionately high relative to the small quantities added. A number of trace metal elements play critical roles in the regulation of metabolic pathways and the activity of certain enzymes and signal molecules.

In CHO cell culture, copper deficiency can result in down-regulation of lactate dehydrogenase and other mitochondrial oxidative enzymes, thus leading to histotoxic hypoxia, regardless of dissolved oxygen concentration.[116] Accordingly, relatively high-copper concentration can shift the lactate metabolism of CHO cells from net lactate production to net lactate consumption, and subsequently enhance cell growth and titer.[117,118] However, high copper concentration can also increase the relative amount of basic variants of an antibody product.[119] Therefore, copper concentration in a CHO cell culture medium should be carefully optimized with respect to both culture performance and product quality.

Iron is widely recognized as a necessary component in chemically defined media. Iron plays a critical role in oxygen transfer through hemes, mitochondrial oxidation pathways, and other important enzymes.[120] However, free iron, especially free ferric ions, can contribute to high oxidation stress even in trace amounts.[121] Iron carriers or chelating agents may be included in the media to minimize toxicity and improve iron uptake by the cells. Transferrin is a very effective iron carrier, which can significantly affect iron uptake.[122] In serum-supplemented media, transferrin is usually present as a component of serum, while in serum-free media, recombinant transferrin can be added to improve iron stability and uptake.[123] As less expensive alternatives to transferrin, small molecule chelating agents, such as tropolone, citrate and selenite, are also used.[110] Tropolone has not been widely utilized or evaluated in CHO-based production due to IP restrictions. Selenite, at an optimized ratio with iron and citrate, has been shown to enhance CHO cell growth in iron-carrier-deficient media by improving iron uptake, and can provide a comparable effect to tropolone, while citrate ion alone without selenite addition did not demonstrate the same effect.[124] However, later studies showed that a combination of ferrous sulfate and sodium citrate can enhance translational efficiency and titer.[125]

Zinc has been reported to be one of the most influential trace elements for mAb yield in chemically defined and protein-free CHO cell culture media. Zinc supplementation in commercial, chemically defined media has been shown to provide a 1.2-fold enhancement in mAb production.[126] Additionally, zinc exposure for CHO cells can result in the induction of stress protein function, and subsequently reduced apoptosis.[127]

Besides the major trace elements discussed above, other elements can also affect cell growth, productivity, and product quality. Most of these trace elements are only required at concentrations below 5 μM in cell culture media. Manganese, molybdenum, selenium, and vanadium are known to be required for cell culture, and are therefore included in most media. Other trace elements, including germanium, rubidium, zirconium, cobalt, nickel, tin, and chromium may be required by certain mammalian cells or have certain functions, and thus are also included in certain media.[2,7] The major functions of selected trace elements in CHO cell culture media are provided in Table 2.

Despite the critical importance of providing trace elements in CHO cell culture media, most trace elements can be toxic to CHO cells at relatively high concentrations.[128] The contamination of media powders, other raw materials, or cell culture

**Table 2. The Function of Major Trace Elements in CHO Cell Culture Media**

| Trace Element | Function | Representative Concentration |
|---|---|---|
| Copper | Supports mitochondrial oxidative enzymes; Regulates lactate consumption[116] | 0.8–100 μM[117] |
| Iron | Impacts glycosylation macroheterogeneity of product[224]; Supports cell growth and health[125] | 10–110 μM[225] |
| Zinc | Enhances mAb productivity[126]; Reduces cell apoptosis progress[127] | 3–60 μM[126] |
| Manganese | Improves galactosylation and reduces sialyation of product[226–228] | 0.4–40 μM*[226] |
| Molybdenum | Increases the production of physiologically active substances[229] | 0.001–0.1 μM[229] |
| Selenium | Facilitates iron delivery; Protects cells from oxidative stress and free radicals[124] | 0.005–0.5μM[124,230] |
| Vanadium | Mimics metabolic function of insulin or insulin analogs and enhances cell growth[15,231] | 0.1–70 μM[231] |
| Cobalt | Increases the N-Glycan galactosylation of product and terminal protein glycosylation[232]; Enhances productivity in chemically defined media[230] | 0–50 μM[230,232] |

*The manganese concentration depends on glycosylation requirement

containers with certain trace elements due to the presence of impurities or improper manufacturing may lead to performance variability or toxicity to cells, if the concentration of those trace elements are borderline in the medium.

### *Salts*

Salts play important chemical and biological roles in CHO cell culture media, including maintenance of cellular membrane potential, osmolality, and buffering.[7] Generally, the bulk ions added to CHO media include sodium, potassium, magnesium, calcium, chloride, phosphate, (bi)carbonate, sulfate, and nitrate.

All mammalian cells use the sodium and potassium ion gradient to generate transmembrane potential, supporting signal transmission as well as nutrition and ion enrichment. In many commonly used CHO cell culture media, the ratio of sodium to potassium ions ranges approximately 20–40: 1.[129] Meanwhile, media with a lower sodium to potassium ratio (6–8: 1) or a relatively high potassium concentration were reported to be beneficial for viability improvement and productivity enhancement with CHO cells.[130,131]

The concentrations of calcium and magnesium ions in media are usually designed at approximately 1–3 mM and 0.2–1 mM, respectively. Absence of calcium and magnesium has been demonstrated to trigger apoptosis via the scavenger receptor BI in CHO cells, while an overload of intracellular calcium may also lead to apoptotic cell death.[132]

Phosphate is a critical anion in signal cascade, energy transfer, and in the formation of many cell components, such as nucleic acid. Phosphate addition in feed media has proven beneficial for cell viability and viable cell density.[133] Adaptation to long-term phosphate deficiency during inoculum expansion media has been shown to lead to a selection of alternate pathways for energy generation.[134]

In addition to their physiological utility, salts are also supplied to control medium osmolality. Ozturk and Palsson reported that increasing osmolality from 290 to 435 mOsm reduced cell growth of a hybridoma cell line, but increased the specific productivity by more than twofold, resulting in a similar final antibody concentration.[135] For CHO cells, specific cell growth rate has been reported to decrease linearly with increasing expansion medium osmolality between 316–450 mOsm/kg.[136] However, gradual increase of osmolality up to ~450 mOsm/kg has also been shown to significantly increase specific productivity and mAb production in a CHO cell process.[137] Therefore, when designing cell culture media, the final osmolality should be taken into consideration in relation to the overall salt and bulk ion supply.

### *Growth Factors*

Growth factors are typically peptides, small proteins, and hormones, which act as signal molecules influencing cell growth, proliferation, recovery and differentiation. Early research showed that growth factors are an indispensable part of cell culture media, without which cell growth may be dramatically inhibited or even stopped.[138] In many early media, growth factors were supplied in the form of serum. In serum-free media, however, instead of supplying the broad spectrum of growth factors that are typically found in serum, only a small number of specific growth factors are supplied, minimizing the overall complexity of the medium formulation.

Insulin and its analogs are among the most widely used growth factors in serum-free media. Studies have shown that trace amounts of insulin as low as 50 ng/ml can enhance titer by 3–4-fold compared to media lacking growth factors.[139] One microgram per milliliter insulin has been demonstrated to improve CHO cell health in batch culture, and to inhibit the expression of apoptosis markers such as ICE, Bcl-2, and Bax.[140] Analogs of insulin, including insulin growth factor 1 (IGF-1) and LONG $R^3$ (Repligen, SAFC), have demonstrated similar or better improvement in CHO cell viability in serum free media, with a lower concentration of the analog required in the medium than insulin.[139,141]

Besides exogenous insulin and insulin analogs, eight autocrine growth factors of CHO cells have been identified at both transcriptomic and immunochemical levels. These include brain-derived neurotrophic factor (BDNF), fibroblast growth factor 8 (FGF8), growth regulated α protein (CXCL1), hepatocyte growth factor (HGF), hepatoma-derived growth factor (HDGF), leukemia inhibitory factor (LIF), macrophage colony stimulating factor 1 (CSF1), and vascular endothelial growth factor C (VEGFC). Studies have indicated that addition of one or more of the growth factors FGF8, HGF, and VEGFC can promote cell proliferation in serum-free media.[142]

However, to minimize the complexity, variability and cost of media, an excessive supply of growth factors exhibiting little effect on cell growth and productivity should be ruled out.[143] One possible alternative to the use of growth factors is the small molecule antioxidant chelator aurintricarboxylic acid (ATA), which has been shown to promote CHO cell growth by acting on IGF-1 receptors (in combination with lysophosphatidic acid) in a manner similar to that of insulin. ATA could therefore

prove useful as a more stable and less expensive alternative to insulin or insulin analogs in certain media.[144]

### *Polyamines*

Polyamines are ubiquitous molecules in mammalian cells, which play key roles in multiple metabolic processes, including DNA synthesis and transcription, ribosome function, regulation of ion channels, and cell signaling.[145,146] In mammalian cells, several polyamines are synthesized from ornithine, a metabolic intermediate in the urea cycle.[147] Regardless, supplementation of culture media with polyamines is essential to support and expedite CHO cell growth.[148]

Exogenous polyamines, which are commonly supplemented in CHO cell culture media, include putrescine, spermidine, and spermine. Putrescine is the precursor for the synthesis of both spermine and spermidine, while spermine and spermidine are interconvertible in cells.[149] Previous studies have shown that each of these polyamines can individually enhance cell growth rate and viability, and that spermine is likely the most efficient of the three.[150] Many CHO cell culture media contain several different polyamines. Meanwhile, polyamine catabolism can generate oxidative species, aldehydes, acroleins and ammonia via multiple pathways, which can inhibit cell growth and ultimately affect cell viability.[151,152] Therefore, polyamine supplementation in cell culture media should be controlled below the limit of cytotoxicity.

### *Non-nutritional components*

In CHO cell culture media, there are some non-nutritional components included to provide a more stable physical or chemical environment for the cells. These components, which may include buffers, surfactants, and antifoam, can have a significant effect on cell growth and productivity.

Buffers are an important part of cell culture media. Although amino acids and multivalent ions can provide some buffer capacity, cell culture media still require strong buffering reagents in order to meet the challenge of maintaining pH in a CHO cell culture. Many traditional cell culture media, such as DMEM and RPMI-1640, applied a bicarbonate buffering system ($CO_2$/$NaHCO_3$) as the major buffering source.[153] However, for increased pH stability, organic zwitterion buffers such as HEPES are also included in cell culture media to provide strong buffering capacity in the pH range of 7.2–7.4.[154] Additionally, phosphate ions included in the medium can also provide a major source of buffering capacity.[2]

Pluronic F-68 is a surfactant commonly included in culture media, which can protect cells from mechanical stress caused by bubbles bursting, by reducing the concentration of cells in the foam layer. Studies have shown that addition of 0.03 g/L Pluronic F-68 in the medium reduces the concentration of cells in the foaming layer to only 30% of the cell concentration in the bulk culture.[155] Pluronic F-68 may protect cells by one or several mechanisms, including formation of a protective layer on the cell membrane, which reduces hydrophobicity, stabilizing the foam layer at the top of the cell culture in the bioreactor, or by incorporation into and strengthening of the cell membrane.[156,157] In general, it is widely agreed that Pluronic F-68 can significantly enhance cell growth, cell viability, productivity, and glycosylation.[158] However, CHO cells can internalize Pluronic F-68 and degrade it in lysosomes.[159] Therefore, in addition to the inclusion of Pluronic F-68 in the basal medium, continuous supplementation of this surfactant as a component of feed medium may be necessary to maintain the desired concentration in the cell culture. Additionally, Pluronic F-68 lot-to-lot variation has been shown to affect cell growth and productivity profiles, especially in large-scale manufacturing campaigns. Peng et al. reported a robust shake flask-based scale-down model to provide a high-throughput solution for quality control and verification of Pluronic F-68.[160]

Antifoam agents are strongly hydrophobic surfactants with low surface viscosity, which are commonly added to cell culture media to control and minimize foaming.[161,162] This is necessary both to protect cells against mechanical shear resulting from foaming, and to prevent the potential "foaming out" of bioreactors and clogging of exhaust filters, which can result in overpressurization. Antifoam may be added directly to the production medium or added on demand during the production process. Antifoam agents may be oil-based (organic) or silicone-based, and can be classified into fast and slow-acting antifoams based upon the defoaming mechanism. Fast-acting antifoams are usually aqueous suspensions or emulsions, of which particles or liquid drops in the emulsion enter the foam bubble film to break bubbles. Oil-based antifoams act more slowly to prevent and eliminate foaming.[163] In CHO cell culture, silicone-based antifoams such as Antifoam C (Sigma-Aldrich), which are typically supplied as aqueous suspensions of silicone polymers, are widely used due to their high defoaming efficiency and low toxicity.[164]

It should be noted that addition of surfactants in general, including Pluronic F-68 and antifoams, tend to reduce oxygen transfer in a bioreactor environment, resulting in lower kLa values.[165,166] Antifoam has been shown to decrease hydrodynamic and mass transfer characteristics in bioreactors, especially with respect to oxygen transfer.[167] Furthermore, relatively high concentrations of antifoam can be toxic to cells, potentially accelerating cell death.[168] Accordingly, antifoam addition should be carefully controlled and kept to the minimum necessary concentration in cell culture.

## Optimization of Growth Media for recombinant protein expression

The development of a robust cell culture manufacturing process for a biologic molecule demands a cell culture medium which not only contains all of the required nutrients for growth and protein expression, but also a formulation which is optimized with regards to cellular health and viability, metabolism, product quality, manufacturability, regulatory considerations, supply chain, and intellectual property (IP). An ideal cell culture medium for a given process or product will optimally address these aspects, while also supporting high protein titer and specific productivity. For these reasons, thorough optimization of mammalian cell culture media is an extremely complex and resource intensive process, as a large number of experiments are necessary to optimize the concentrations of a large number of medium components with respect to so many desired attributes of the final formulation and performance of the media.

### *Design elements of an optimized medium for industrial applications*

Industrial cell culture processes require media that are amenable to consistent large-scale manufacturing and preparation. As such, dry powder and liquid media formulations should be optimized for attributes affecting manufacturability, such as

component solubility, media filtration, sterilization, storage stability, media preparation scalability, and raw material consistency with minimal lot-to-lot variation. Media optimization efforts for fed-batch processes typically focus on nutrient feed development for improved growth and productivity. However, for industrial applications, manufacturability attributes and engineering concerns such as preparation time, shear protection, and foam reduction are equally important.

As opposed to a "batch" process, in which cells are grown in a single culture medium until nutrients are depleted, a "fed-batch" process comprises at least two media: a basal medium to support initial growth, and one or more feed media. Feed media may be added as a bolus at specified intervals, or continuously later in the process, to replenish depleted nutrients, supporting stationary phase growth and protein expression. In its simplest form, a feed medium may supply only glucose, the primary carbon source needed to provide energy for cellular growth. More commonly, feed media resemble a concentrated version of the basal medium, up to 15 times more concentrated with respect to certain components of the basal.[169] Feed media may be designed based on data from batch culture experiments, which determine nutrient consumption and depletion rates. Nutrients are separated into basal and feed media, in order to provide the nutrients to the cells at optimal levels without reaching solubility limits or inhibitory concentrations. Certain nutrients, such as glucose, may be fed separately in order to control concentration in the media more precisely, and to prevent accumulation of unwanted metabolites such as lactate.[7]

In an industrial setting, it may be undesirable to use a different medium during early stages of cell line development, such as transfection and single cell cloning, than the basal medium that is used later for inoculum train and basal production medium. While use of a different medium up front may allow for the use of commercially available media already optimized for sensitive steps such as transfection or cloning, it may nonetheless require lengthy periods of adaptation to richer basal and fed-batch media formulations. Adaptation steps can then extend project timelines and expose cells to additional pressure, possibly resulting in selection of unstable subpopulations. An ideal cell culture process would utilize a single medium from transfection and cloning through to production basal medium, requiring little to no adaptation, and with only minor differences between cloning and basal media (supplemental growth factors, etc.)

As previously mentioned, most modern cell culture manufacturing processes utilize chemically defined, serum-free and animal-component free culture media. This addresses issues around potential contamination with adventitious microbial or viral agents arising from serum or animal components, as well as consistency and reproducibility of undefined media components such as hydrolysates. While chemically defined media have been in use for some time, and many effective formulations are readily available, further optimization of the medium is usually required to develop a robust, high titer process for a specific clone. Growth, metabolite, and nutrient depletion profiles can vary between clones from a single transfection. Therefore, optimization efforts are directed at increasing titer and also at maintaining needed nutrient levels, optimizing trace elements, salts, and osmolality, minimizing accumulation of waste metabolites, preventing apoptosis and viability crash, and avoiding cellular aggregation or clumping.

Manufacturability is a critically important characteristic of optimized media for industrial cell culture processes. Mammalian cell culture media are very complex, often including greater than 50 components. In an industrial setting, this complexity makes it challenging to ensure scalable and reproducible media preparation, providing consistent performance from small-scale process development through large scale clinical and commercial manufacturing. As such, it is now common practice to prepare a milled and blended "premixed" dry powder formulation, containing all or most of the components. Milling is performed using different types of milling equipment, such as a ball mill, pin mill, or FitzMill (The Fitzpatrick Company, Elmhurst, IL), depending on the manufacturer. The premixed powder can be manufactured reproducibly in small scale and large-scale quantities, is easily shipped, has an acceptable shelf life, and typically only requires reconstitution with water followed by pH adjustment and filter-sterilization prior to use. Liquid media formulations or concentrates can also be provided by media manufacturers, but these may be costly to ship in large volumes and have shorter shelf lives.

For dry powder formulations, due to solubility limits of certain components, it may be necessary to divide the overall formulation into multiple premixed powders. Typically, a medium comprised of 1–2 premixed powders is ideal for minimizing the complexity of media preparation. Additional components may be added individually or in the form of supplements, depending on the attributes of the component in terms of solubility, stability, and sterilization requirements. Optimization of media formulation should take into account raw material availability (dual or multisourced), solubility, osmolality, scalability, stability at room temperature, wettability of dry powder components, and consistency of the final milled and blended dry powder or reconstituted liquid medium. Certain powder manufacturing processes exist, such as the Gibco Advanced Granulation Technology (ThermoFisher), which promise easy and reproducible media preparation by use of a complete media powder which is already pH and osmolality adjusted, and requires only reconstitution with water and filter-sterilization prior to use. Alternatively, MilliporeSigma has developed a media powder compaction technology, which can be used to optimize granule size for improved media handling and dissolution with minimized dust formation.[170] However, in a manufacturing setting, it is critical to have multiple suppliers for all media powders and components, in order to mitigate potential supply chain issues. The use of a proprietary powder formulation may restrict sourcing to a single supplier.

Certain media components have well-known liabilities concerning stability and solubility. Changes in nutrient availability and redox balance can result from the degradation or precipitation of media components. Liquid media are typically kept cold and protected from light during storage, to prevent degradation of light sensitive or heat labile components. Several amino acids, including Glutamine, Tyrosine and Cysteine have stability and/or solubility issues that limit the ability to provide these components at the necessary concentrations in the media.[2,106] Additionally, certain growth factors, including insulin and IGF, as well as many vitamins, may be unstable in liquid media.[2,171] Media can be designed to overcome these limitations by including unstable components, such as Glutamine and growth factors, in the form of separate supplements, which are added just prior to use, and continually supplied as part of the feed medium to avoid depletion. Amino acids may also be supplied in the form of small peptides or dipeptides to provide higher concentrations in the culture media with improved stability and solubility.[2,106]

Osmolality is a major concern in culture media design. All ions, including salts, amino acids, buffers and fatty acids, can

contribute to osmolality. High osmolality (>450 mOsm/kg) can result in decreasing cell growth, titer, and cell viability, as well as increased cell size and doubling time.[136,172] Below 450 mOsm/kg, increasing osmolality has limited direct influence on cell growth. In fact, in the production phase, higher osmolality (up to approximately 400 mOsm/kg) has been shown to enhance titer by >20%.[173] However, increasing osmolality up to 450 mOsm/kg can also lead to higher production of ammonium and lactate, thereby indirectly affecting culture performance. Therefore, production and expansion media for recombinant protein expression in CHO cells are usually designed to maintain relatively low osmolality (250–350 mOsm/kg), while feed media increase osmolality in the late phase of a production process.

Engineering concerns are also a critical aspect of medium design. Cells are damaged by shear stress introduced by mechanical agitation, sparging, and foaming.[161] This is especially pronounced in chemically defined and serum-free media, likely due to their overall low protein content.[156,157] Foaming is therefore a significant concern in bioreactors for multiple reasons. In addition to its negative effect on cell viability and productivity, in severe instances, foaming can be catastrophic to a process by leading to clogging of exhaust filters, over-pressurization, and contamination. As described previously in this review (section Polyamines), protective polymers and surfactants, such as Pluronic F-68 and antifoaming agents, are routinely included in chemically defined media to reduce shear-related damage and control foaming. Optimization of Pluronic and antifoam levels in a medium can help to maximize shear and foam protection while minimizing negative impacts to oxygen transfer and kLa values in a bioreactor environment.[165,166]

Virus control at the point of medium preparation is another significant manufacturability concern. Inactivation methods employ ultraviolet (UV) irradiation, gamma irradiation, heat, extremes in pH, or solvent/detergent exposure. For chemically defined media, ultrafiltration and high temperature-short time (HTST) treatments have been used to reduce the risk of viral introduction. HTST, while effective, can lead to precipitation or formation of insoluble particles in cell culture media. It has been shown that adjustment of calcium salt and inorganic phosphate salt concentrations in the basal medium formulation led to substantial reduction in precipitation due to heat treatment[174] Another approach is the use of viral-retentive filtration, which is broadly applicable and may require minimal special considerations for chemically defined medium.[175]

### *Experimental approaches to medium optimization*

Once the key design elements of the desired cell culture media have been identified, there are several different and potentially complementary approaches to medium optimization that can be taken.[176] Essentially, the specific concentration of each component of the medium should be optimized with respect to its rate of utilization by the cell line, and its effects on titer, growth, and product quality. Interactions between multiple media components must also be taken into consideration, which can complicate optimization efforts using a simple, empirical one-factor-at-a-time (OFAT) approach.[2]

Many of the essential media components described previously, including glucose, amino acids, vitamins, lipids, and fatty acids, are consumed in a stoichiometric manner during cell growth, as they are either converted to biomass and recombinant product, or else they are utilized as energy sources.[7] These nutrients can therefore be optimized stoichiometrically, by analysis of spent cell culture media and calculation of specific utilization rates for each component.[177] Depletion of amino acids, trace elements and vitamins can be monitored using various analytical methods, in order to optimize the concentration of these nutrients in the media, improving growth, and prolonging viability. A stoichiometric model for mammalian cell growth and monoclonal antibody expression has been developed,[19] and successfully utilized to optimize glucose, glutamine and amino acid concentrations in cell culture media and supplements, resulting in significant improvements in cell density and product titer.[18,20,21] Although initially developed for hybridoma cultures, this stoichiometric model has also been applied to a CHO cell process for recombinant Gamma-interferon expression, resulting in improved growth, production and glycosylation of the final product, with reduced accumulation of metabolic byproducts in the culture medium.[17] These studies demonstrate the general applicability of stoichiometric models for medium optimization.

While stoichiometric nutrient balancing based on consumption and depletion rates can be an effective approach to medium optimization, it does not permit in depth observation and understanding of the flow of nutrients through complex cellular metabolic pathways. Metabolic Flux Analysis (MFA), on the other hand, aims to account for the major pathways and biochemical reactions comprising cellular metabolism by measuring the flux rates of nutrients and metabolic intermediates through individual pathways during growth and production phases of cell culture.[36,177,178] Material balance reactions are challenging to apply to complex cellular metabolic systems, in which a large number of reactions occur, including some with very low flux, which may be difficult to quantitate. In such cases, the overall analysis can be reduced to contain only the most significant and well-characterized pathways, with measurable fluxes that can account for the majority of glucose and amino acid metabolism and conversion to biomass and product.[178]

The stoichiometric analyses mentioned previously which defined key metabolic and nutrient demands involved in animal cell growth and protein expression, and the eventual application of a stoichiometric model to a CHO cell culture process[17–20] led the way for the future development and application of MFA for CHO cells. MFA has been used to model central metabolism in CHO cells,[179] and has been applied to CHO cell culture processes to gain insight into glucose and nitrogen utilization,[63] and lactate consumption.[180] More recent studies have established very complete metabolic models for CHO cells, paving the way for easier media and process development using in silico models to predict cellular response under modified nutrient conditions.[181]

### *Experimental design and application of high-throughput technologies for medium optimization*

Because cell culture media are highly complex, containing greater than 50 components in many cases,[5] the optimization of a medium formulation can be very challenging. The traditional, one-factor-at-a-time (OFAT) approach to media optimization is very labor and resource-intensive for such complex formulas. As this method only varies the concentration of one component at a time, while keeping all others constant, such an effort would require a large number of culture vessels and significant personnel hours to perform.[81,182] Furthermore, this method

| | Pattern | X1 | X2 | X3 | X4 | X5 |
|---|---|---|---|---|---|---|
| Test 1 | +++++ | 1 | 1 | 1 | 1 | 1 |
| Test 2 | +−+++ | 1 | -1 | 1 | 1 | 1 |
| Test 3 | −+−++ | -1 | 1 | -1 | 1 | 1 |
| Test 4 | −− +−+ | -1 | -1 | 1 | -1 | 1 |
| Test 5 | +−−−+ | 1 | -1 | -1 | -1 | 1 |
| Test 6 | −− +−− | -1 | -1 | 1 | -1 | -1 |
| Test 7 | +−−+− | 1 | -1 | -1 | 1 | -1 |
| Test 8 | ++−−− | 1 | 1 | -1 | -1 | -1 |
| Test 9 | +++−− | 1 | 1 | 1 | -1 | -1 |
| Test 10 | −+++− | -1 | 1 | 1 | 1 | -1 |
| Test 11 | −−− +− | -1 | -1 | -1 | 1 | -1 |
| Test 12 | −+−−+ | -1 | 1 | -1 | -1 | 1 |

**Figure 1. Example of a Plackett-Burman Design for medium optimization, for a DoE of five medium components and 12 tests.**

cannot identify interactions between the components, and therefore has limited potential for medium optimization.[71]

The use of statistical design of experiments (DoE) has been demonstrated to streamline and improve this effort, by evaluating multiple components and their interactions at once, reducing the necessary size and number of experiments, and allowing observation of complex interactions between medium components. Many different statistical designs exist, each with its own distinct advantages. Designs range from large, full factorial designs in which several factors are evaluated at two or more levels, in all possible combinations, to fractional factorial designs in which only a subset of combinations is evaluated, to more sophisticated models such as Plackett-Burman, Central Composite, and Box-Behnken, which can identify major effects and some interactions while reducing the overall number of experiments required.[183] Plackett-Burman designs (Figure 1), in particular, are very efficient for screening a large number of factors while minimizing the number of experiments. However, results may be confounded by two-factor interactions, thus requiring the use of different designs to optimize further once the key factors and main effects are identified.[183] Early examples of the use of statistical DoE for CHO cell culture media developments utilized Plackett-Burman style screening designs to develop formulations for improved growth and recombinant protein expression in serum free media.[2,184,185] Since these early efforts, use of statistical designs has become routine for cell culture media optimization, with many groups combining a Plackett-Burman type design for the initial screening of components for major effects and interactions, with a central composite or custom design to zero in on the optimum formulation in subsequent experiments (Figure 2).[71,182,186–189]

Media mixing (or blending) is another approach which has been used to simplify the optimization of complex cell culture media components, while also allowing for the evaluation of individual components and their interactions. By this approach, multiple media differing in overall composition or in the relative concentration of specific components (or groups thereof ), are mixed in varying ratios to generate a larger number of media "blends," which can then be screened for their effect on cell growth and recombinant protein expression.[177] As described previously, this approach was used during some of the earliest efforts to develop mammalian cell culture media, and was responsible for the creation of important breakthrough formulations such as DMEM/F12 and RDF.[177,190] Media blending is still used in optimization studies in

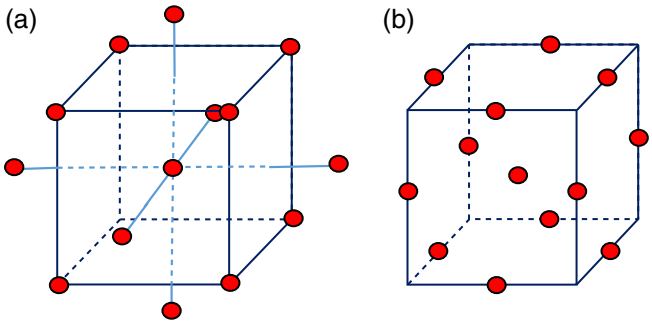


**Figure 2. Examples of Central Composite and Box-Behnken experimental designs. (a) Example of a Central Composite Design for medium optimization, for a DOE comprised of 3 medium components and 15 tests. (b) Example of a Box-Behnken Design for medium optimization, for a DOE comprised of 3 medium components and 13 tests.**

order to minimize solubility, osmolality and pH issues arising from the use of component stock solutions to generate experimental media for screening.[190] Blended media can be evaluated and directly compared in order to identify blended formulas, which offer improvements over any of the parent media, or specific components whose relative concentrations in different blends demonstrate a significant effect on culture performance. Furthermore, statistical experimental designs and analyses can also be applied to media blending experiments to predict optimal formulations not specifically represented in the set of media blends tested. These approaches have been successfully used to optimize amino acid, hexose sugar and other component concentrations in CHO cell culture media,[190,191] and also in the development of commercially available kits for medium optimization.[192]

Even using the simplified and streamlined experimental designs just discussed, successful optimization of mammalian cell culture media still requires the capability to run and evaluate a large number of culture vessels concurrently. This is necessary to enable studies of sufficient size to evaluate multiple component ranges with experimental replicates to ensure robustness. Shake-flasks can be replaced with smaller-scale shaking vessels such as 50 ml conical tubes or deep-well plates (DWPs) to minimize required shaker space, which can also be paired with automated liquid handling to minimize manual labor associated with sampling and feeding.[193] The SimCell micro-bioreactor system, developed by BioProcessors Inc., contained an array of 600 µl bioreactors, which could be run in large numbers, with up to 1000 reactors running in parallel, to conduct very large cell culture experiments for medium and parameter optimization. This system was evaluated through collaboration with Amgen, showing comparability and scalability to 5 L systems, and the platform was eventually purchased by Novo Nordisk.[194] More recently, the advent of fully automated, small scale bioreactors such as the Ambr systems (Sartorius) has enabled high throughput optimization of cell culture processes in a true bioreactor environment, with internal impellers, gas supply, pH and dissolved oxygen control as well as in-line cell counting.[195,196] These types of technologies are extremely useful and valuable tools, which greatly enhance the researcher's ability to properly and thoroughly evaluate and optimize complex media formulations.

## Recent Advances in Medium Development

Modern approaches to CHO cell culture media development have leveraged new or existing technologies to drive increased

performance, by directly addressing issues of cellular metabolism, genetic makeup, and signaling pathways that can inhibit growth and productivity or even lead to premature cell death via mechanisms such as apoptosis. New technologies can support automated and aseptic sampling of bioreactors, as well as real time analytics and diagnosis via Process Analytical Technology (PAT) approaches such as capacitance and Raman spectroscopy, which allow development of feedback loop adjustments to medium composition. Advances in "multiomics" technologies, incorporating data from genomic, epigenomic, transcriptomic, proteomic, metabolomic, and other analyses, enable an integrated and complete picture of metabolism and its control. Other drivers for recent advancements in media design have included the use of high-throughput cell culture equipment and methodologies to decipher and control product quality, particularly in the context of matching product quality for biosimilars, as well as the need to sustain high cell densities for long periods to enable continuous processing. Directly addressing these issues can lead to the development of highly customized media, optimized for specific cell lines and processes to maximize cellular productivity.

In recent years, perfusion technology has been increasingly applied to seed train (N − 1) and/or production stage bioreactors to increase cell growth and productivity, improve the efficiency of bioreactor usage, and reduce manufacturing cost.[197–199] By continually removing spent media containing toxic metabolic byproducts, while also continually feeding fresh media at a comparable rate, perfusion processes can eliminate issues of nutrient depletion and growth inhibition if the perfusion medium is properly designed (Figure 3). Unlike regular batch or fed-batch processes, the viable cell density in a perfusion process can reach as high as 20–35 million cells/ml with cell bleeding, and 200 million cells/ml without cell bleeding.[200] Therefore, perfusion media need to be specially designed to support very high cell densities and growth rates.

Despite differences among the various perfusion operations which are utilized for recombinant protein expression in CHO cell culture, including N − 1 perfusion, concentrated fed-batch and continuous perfusion production, the starting point for perfusion medium development is typically the basal and feed media used for fed-batch culture. One successful case of perfusion medium development, which resulted in a titer of 1.2 g/L per day, was achieved by optimizing the ratio between existing basal and feed media, removing unnecessary components, increasing medium depth, optimizing osmolality, and rebalancing amino acids and vitamins.[201] However, more so than with fed-batch media design, logistical issues such as simplicity of operation and compatibility with manufacturing facilities must be taken into account in the development of perfusion media. In particular, lower perfusion rates are highly desirable in perfusion processes, as they require smaller volumes of perfusion media. As such, this should be prioritized in perfusion medium development. Concentrated media formulated for precise delivery of nutrition can facilitate the manufacturing process and reduce cost.[202] Although perfusion experiments and processes are typically executed in bench-scale bioreactors,[203] inexpensive and convenient scale-down models for perfusion have been developed in deep well plates, spin tubes, and automated bioreactors, which can enable the large statistical DoE screens required to optimize perfusion media.[201,204,205]

Genomic and transcriptional profiling methods have also been used to evaluate CHO cell culture processes and identify areas for medium improvement. Schaub and colleagues compared high-titer and low-titer fed batch processes for the same mAb-producing cell line, and identified significant differences in gene expression resulting from differences in media formulation, with specific effects on metabolic pathways affecting cell growth and viability.[29] Furthermore, the identification of differential expression of genes related to lipid metabolism between the two media allowed the targeted optimization of an existing media formulation based on transcriptomic data, resulting in a 20% improvement in titer in that medium.

Metabolomics approaches may also be useful in optimizing media formulations. Miranda Yap and colleagues have utilized HPLC-MS to characterize intracellular and extracellular metabolites derived from amino acids, media components, and various metabolic pathways, which can accumulate in the media during cell culture, inhibiting growth or triggering apoptosis.[206–208] Additionally, Mulukutla and colleagues have used mass spectrometry and NMR to identify and quantitate metabolic byproducts, which were accumulating in a fed-batch process and inhibiting growth, despite good control of lactate, ammonia and osmolality in the process.[32] These growth-inhibitory molecules were identified as shunt (or dead-end) byproducts of the metabolism of certain amino acids, including phenylalanine, tyrosine, tryptophan, methionine, leucine, serine, threonine, and glycine. The authors demonstrated that careful control of amino acid levels in the cell culture, by feeding at a rate which matches the specific consumption rate, can control inhibitor accumulation, and improve growth and productivity.

While genomic and metabolomic approaches have already demonstrated utility in improving CHO cell culture processes, greater advances may be realized by integration of these techniques along with other "omics" analyses, including proteomics, glycomics, epigenomics, fluxomics, lipidomics, and transcriptomics, to build more complete mechanistic models.[209] A very large study with academic and industrial contributors from multiple countries has developed a genome-scale metabolic model of the CHO cell, and demonstrated its utility for optimizing growth and productivity, and for determining useful targets for genetic engineering to improve recombinant protein expression and secretion.[210] Furthermore, the authors developed cell line specific models for CHO-K1, CHO-S, and CHO-DG44 lineages by incorporating transcriptomic, metabolomic, and proteomic data for each cell line with the genome scale metabolic model.[210] With the continued advancement of omics-enabling molecular and analytical technologies, models such as these can be improved further through incorporation of glycomic, proteomic, and epigenomic data to model product quality and cell line stability as well as metabolic levers to control growth and productivity through media development for a given CHO cell line.[209] Application of these types of advanced analyses to development of a CHO cell process can potentially enable highly specialized media development, tailored to a specific cell line based on its own unique "multiomic" profile.

Certain molecules which target cellular pathways and mechanisms, involved with cell health or protein expression can be added to cell culture media. Apoptosis and Autophagy are programmed cell death processes, which can be triggered by nutrient depletion or environmental stress, potentially decreasing overall cell health and cell culture performance. Supplementation with apoptosis inhibitors[211–214] and autophagy inhibitors[215,216] has been evaluated, in some cases improving recombinant protein expression. Most of these supplements are not widely used due to the cost at manufacturing scale.

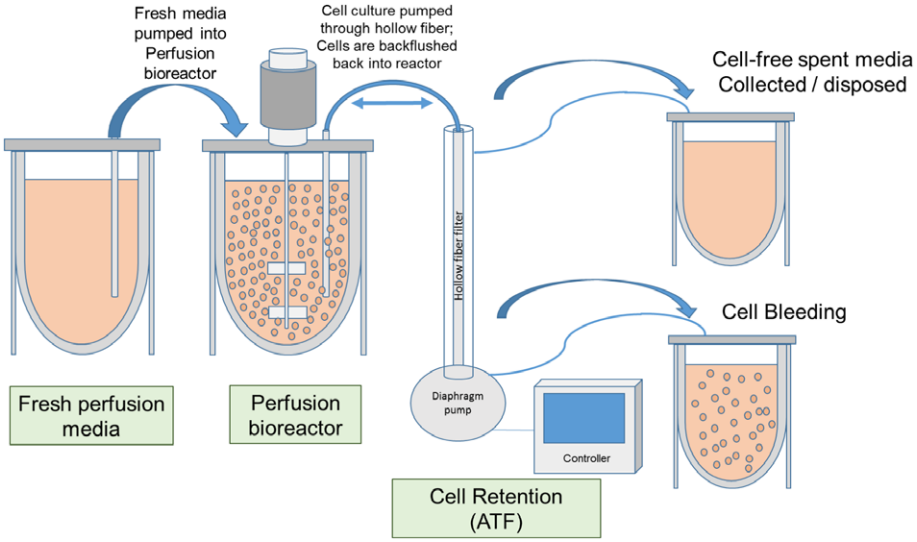


**Figure 3. Illustration of a perfusion bioreactor system.**

However, certain growth factors that are widely used, such as Insulin, IGF − 1, and LongR3 have been demonstrated to improve growth and productivity by inhibition of apoptosis.[139,140,212] Certain small molecules including sodium butyrate and valproic acid, which are known to inhibit histone deacetylase, thereby enhancing transcription by improving the accessibility of genes, and also to arrest the cell cycle in the G1 phase, increasing the population of productive cells in the culture, have been evaluated for their effect on recombinant protein expression in CHO and other mammalian cell lines.[217–223] While these molecules have been shown to improve specific productivity, they can inhibit growth and induce apoptosis, reducing overall productivity. This can be controlled via the timing and concentration of the addition, as well as strategies to control apoptosis, such as reduced temperature.[218,219]

In addition to media formulation, feeding strategies can play a critical role in improving cell growth and productivity by optimizing the rate of nutrient uptake and minimizing the accumulation of inhibitory waste metabolites. Greg Hiller and colleagues at Pfizer have implemented a glucose feeding strategy (named HIPDOG) triggered by the pH of the cell culture, which minimizes lactate accumulation in the culture, and dramatically improves growth, titer and specific productivity.[30] This method is based on the rise in pH due to lactate consumption when glucose is consumed to a very low level. The pH rise triggers a glucose feed, bringing the pH back down due to glucose consumption and lactate production. Essentially, pH is controlled on the high end of the range by a glucose feed, hence the name Hi-End pH-Controlled Delivery of Glucose (HIPDOG). This clever strategy has also been modified for use with a hybrid perfusion and fed-batch process, in which the rate of perfusion feed is similarly controlled by pH (HIPCOP).[202] Meanwhile, at Genentech, Lu and colleagues have demonstrated the benefit of dynamic feeding strategies, in which highly optimized feed medium is fed at a rate determined either by online capacitance cell density measurement or by automated glucose monitoring, also achieving substantial improvements in titer for certain cell lines.[23] Other studies at Genentech have demonstrated the use of lactate and pyruvate feeding to minimize potentially inhibitory $CO_2$ and Ammonia accumulation in cell culture.[65] Feeding strategies such as these, coupled with fully optimized media formulations, can truly maximize the potential for growth and productivity of a CHO cell culture process.

## Conclusion

Cell culture media have evolved from early formulations containing serum, to complex hydrolysate-containing media, to completely chemically defined and (in some cases) protein-free formulations. Coupled with concepts of rebalancing basal and feed media compositions to optimize nutrient delivery while controlling final osmolality, as well as stoichiometric feeding, cell culture process and medium design have coevolved to maximize performance and consistency. Modern "omics" approaches continue to provide better mechanistic insights, to further develop and optimize media enabling productive and consistent cell culture for biomanufacturing. Better mechanistic understanding should also help in the development of media that will give similar high performance and product quality across multiple cell lines, and help attain larger leaps toward optimal performance.

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