Pharmaceutical BIOPROCESSING

Identifying and eliminating cell culture process variability

Critical to the biopharmaceutical industrial objectives are robust, reproducible processes which result in consistent product quality and yields. These parameters support the product safety and efficacy as well as control over the process and supply chain. To have a consistent cell culture process, process inputs must be reliable. Historically cell culture media have been a source of variability through the inclusion of complex components such as hydrolysates and sera. Industry has shifted to chemically defined basal and feed media and seen reduced variability but chemically defined media have not eliminated process variability. This review will consequently focus on media variability, the subsequent outputs of lactate and ammonia production and product quality, and the possible routes to eliminate process inconsistency.

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The overall goal of the biopharmaceutical industry is to manufacture safe and efficacious proteins with a reliable supply chain, such that the patient always receives the expected drug product. A robust cell culture production process is critical to achieving this objective. To begin, first we define a cell culture production process as all aspects involved starting from a frozen vial containing cells to the harvest of the production bioreactor. Between the frozen vial and the cell culture harvest, there are several stages. First the frozen vial is thawed and fresh cell culture medium is inoculated. After a growth period typically on the order of days, cells are transferred from the first stage to a second stage of a larger working volume. This growth incubation period followed by a transfer into a larger working volume is repeated until sufficient cell numbers are accumulated and typically four to eight growth stages are required. Finally the production bioreactor is inoculated.

Consistency in cell culture production processes depends on the cell line and all of the process inputs. Each aspect must be considered for its overall robustness in order to ensure a consistent process. Impacts on quantity and quality of the process and product are often but not necessarily interrelated. Quantitative aspects are best expressed in form of **volumetric productivity** (Q_p, mass per volume per time), whereas qualitative aspects are represented in the **product quality** (PQ) profile of the drug substance. Both Q_p and PQ are standard abbreviations used within the cell culture industry. The titer of a cell culture process (simply the product concentration in the harvested culture suspension) often serves as a simplified measure for productivity. Variability in the cell line may result in different metabolism, which in turn can negatively affect titer and PQ. Variability in process inputs and controls can have similar negative effects. Understanding the specifics of how each of these variables impact Q_p and PQ can ultimately lead to better control of both parameters. A great deal of **process variability** is inherently dependent on cellular metabolism as metabolism is a major driver for culture pH, metabolite (such as glucose, lactate, amino acids and ammonia) concentrations for example which are all critical for Q_p and PQ. Consequently, the variables that impact Q_p and PQ can be thought of as the variables that impact metabolism. A great deal of work has resulted in very high Q_p without negative effects on PQ,

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Key terms

Volumetric productivity (QP): Refers to the mass of protein produced per unit volume per unit time. The volumetric productivity connects directly to the facility capacity to produce the protein of interest. The typical unit is mg/l/d.

Product quality (PQ): Refers to all of the modifications of the protein, including glycosylation and amino acid modifications such as deamidation. Changes to the PQ profile connect directly to the safety and efficacy of the protein of interest.

Process variability: Refers to the consistency of the process. For the purposes of this review, a variable process is defined as one where the cell culture parameters vary by more than 15% or the PQ is found to be outside of the specifications for that product.

and recent reports have demonstrated high titers up to 10 g/l and Q_p in excess of 500 mg/l/d [1–5]. Innovations in process have been required, including a strong focus on cell culture media optimization.

Cell culture processes that deliver quantitative results with a coefficient of variation of less than 15% are considered well controlled. Some of the common analytical tools used to monitor cell culture processes have approximately this much inherent variation [6,7]. Despite these limitations, when the cell culture process is maintained in such a range, PQ typically falls well within established specifications. Process variability as we define in this review would include variability outside of the 15% window, and also may deliver PQ outside of specifications or at least clearly outside of typical variability of PQ.

To understand variability that is rooted in metabolism, first a basic understanding in metabolism is necessary. Lactate production is a key aspect of mammalian cell metabolism, and there are substantial differences observed between lactate producing versus consuming cultures [8,9]. As metabolism is strongly impacted by the extracellular condition, the cell culture medium plays a key role. Historically hydrolysates and sera were common aspects of industrial processes. Recently chemically defined media have been replacing these undefined components in order to specifically eliminate the lot-to-lot variability observed from these undefined and complex mixtures. Even with chemically defined media, there may still be variability in PQ as even small changes in some media components have been shown to result in altered metabolism and PQ. A logical conclusion would be to further increase control of raw materials that become part of a culture production process. In addition, advanced process control tools may help to further enhance culture performance consistency and robustness against raw material variability. Together advanced process control and enhanced raw material control promises significant improvement of consistency and robustness against interfering variables. In this review, we will address the basics of metabolism, the variability observed using hydrolysates and chemically defined media, the metabolic effects on PQ, and also the routes to limiting cell culture process variability.

Fundamentals of mammalian cell metabolism

Glucose is the main energy source and thus a foundation of most cell culture processes for industrial applications. At a high level, when glucose is consumed for energy production, glucose feeds down the glycolysis pathway and then diverges either to lactate as a waste product or into the tricarboxylic acid cycle. Despite glucose being the main carbon source for metabolism, some processes also include other carbohydrates, either added exogenously or as a contributing factor of a complex media ingredient. Galactose has been added to cell culture processes specifically in order to impact PQ [10–13]. Yet galactose may affect metabolic profiles and specifically lactate production or consumption in certain situations [14–17]. Amino acids are consumed or produced and fed into cell mass and metabolism as well by producing or consuming tricarboxylic acid cycle intermediates such as α-ketoglutarate. When amino acids are consumed, ammonium may be released as a waste product. Ammonium may be toxic [18–20] and may lead to reduced galactosylation and sialylation [21–24] by changing intracellular pH gradients and nucleotide sugar levels [25]. Ultimately this may lead to decreasing expression levels of sialylation-related genes [26]. Figure 1A depicts this high-level overview of metabolism and also demonstrates via the schematic how the metabolic pathways relate to PQ modifications. Figure 1B breaks down product modifications that may occur by chemical and biological mechanisms. Biological mechanisms for product modifications may be intracellular or extracellular. From within the overall context presented in Figure 1, this review seeks to highlight how metabolic pathways may lead to variation in the related PQ attribute.

Consistency in glucose consumption rates will dictate the consistency of the process. Historically many processes exhibited lactate production phenotypes only, similar to the Warburg effect [27–30]. Lactate production directly leads to a decreased pH. Eventually a process will reach the lower deadband for controlling pH, and base will be added. This has the secondary effect of increasing osmolality. Consequently variations in lactate metabolism may impact productivity and PQ through a direct effect of osmolality [31–33]. In addition, variations in pH may impact productiv-

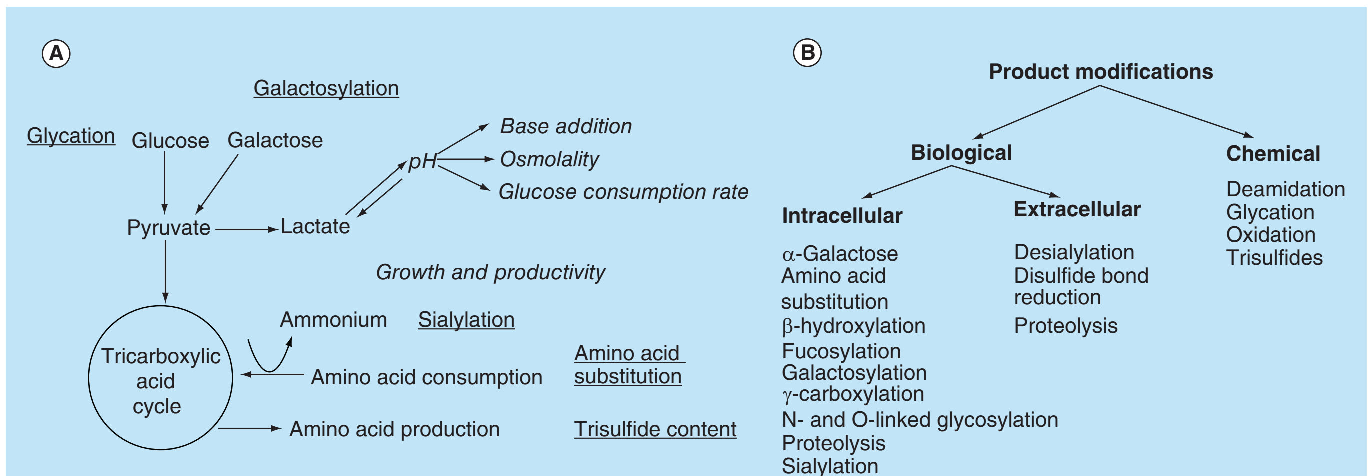

Figure 1. Linkages between cell culture concerns and product quality. (A) High-level overview of the central metabolic pathways important for cell culture. Subsequent effects on cell culture process outputs are highlighted in italics. Subsequent effects on product quality are underlined. **(B)** High-level overview of product modifications are broken down by biological and chemical modifications. Modifications with a biological basis are further broken down as intracellular versus extracellular modifications.

ity [34–37] and can alter PQ as well [38]. The impact of changes in pH can be mitigated by controlling within a small pH range. Yet the benefits must be counterbalanced with the risks of more variable osmolality profiles and additional carbon dioxide accumulation caused by standard acidic control by carbon dioxide delivery.

Lactate metabolism always has been of considerable interest. Much of the recent focus has been on the different extracellular and intracellular conditions required to favor lactate consumption as compared with lactate production phenotypes [3,8,9,39–44], and variability in lactate production has been shown to be linked to overfeeding [45], dissolved oxygen [46], culture temperature and pH [47,48]. Process temperature and DO are well-controlled parameters, but pH may vary substantially during fed-batch culture due to its relationship with pCO2 and other metabolites. At higher culture pH, the increased glucose uptake and lactate production rate are related to increased activities of glycolytic enzymes [49]. The consumption of many amino acids is significantly elevated at higher pH as well. In order to control lactate at very low levels, one novel solution has been to feed glucose as part of high-end pH control strategy, allowing lactate to be consumed and very low levels of glucose maintained to minimize lactate production [42]. Another approach to dealing with lactate production has been to downregulate lactate dehydrogenase [50,51]. As copper levels have been shown to be critical to lactate metabolism [9,40,52] and copper is a trace metal in most media formulations, it is clear that a well-established medium without variation in trace components is required for a robust process.

While it is beneficial to keep lactate at a lower level, increased ammonium has been observed when lactate depleted during fed-batch culture. As ammonium is critical to many PQ attributes, feeding lactate or pyruvate to sustain alanine production may provide the benefits of reducing ammonium accumulation [53].

Defining process variability & sources of variation

With any process, there is an expectation of established performance based on historical data using a suitable number of cultivations. Variation in an individual experiment away from the historical data is comprised of the total variability of the analytical instrumentation and the cell culture process. When analyzing the system, variation in cell culture data must be investigated within the context of both sources of variability. Here we discuss these sources of variation separately and present an example data set for analysis.

Analytical variation

Analytical instrumentation reproducibility is easy to quantify, and there are several reports demonstrating the accuracy of standard cell counting methods and metabolite quantitation. Viable cell density and viability are key process parameters to monitor as differential cell growth and cell death are definitely expected to result in altered metabolism and PQ. It has been shown that standard automated cell counters, such as the Cedex, have sufficient instrument-to-instrument variability that prevents their interchangeable use [6]. In fact, rigorous and daily calibration procedures were required in order to get agreement between cell counters at less than 5%. Prior to those calibrations,

instrument-to-instrument variation was observed at up to 15.8% [6]. This is a critical parameter to understand as it implies that a culture measured at 10×10^6 viable cells/ml is potentially identical to another culture measured at approximately 8.6×10^6 viable cells/ml. Ordinarily one would assume a decrease in cell growth in the latter culture, but instrument-to-instrument variation cannot be ignored. Automated cell counters are certainly improved over manual counts, but difficulty with determining viability for a low viability sample remain [54]. New automated cell counters based on the Coulter principle are recently available with reports of excellent reproducibility [55,56]. For glucose quantification, Bawn and coworkers compared the industrially common, membrane-based technology in the NOVA Bioprofile® 400 with absorption photometric-based technology in the Roche Cedex Bio [7]. The report demonstrates that the membrane-based glucose measurements have a residual SD of approximately 12% while the absorption photometric-based measurements have a residual SD of approximately 5%. Both technologies report glucose concentrations that are not statistically different than each other. For osmolality measurements, the freezing point depression technique has been around for decades and the instruments are highly accurate. Osmometer manufacturers typically report less than 1% variation, and variability in osmolality measurements usually is not of concern.

Biological variation

Biological sources of variation can come from diverse aspects of the process. Briefly, the cell bank used, the seed expansion robustness and the production process control strategy and process design may all impact the production cell culture. As examples, cells cryopreserved during stationary phase are generally accepted to recover after the thaw stage with inferior results as compared with cells frozen during exponential growth. Similarly a seed culture which permits cells to reach stationary phase may not reach the desired transfer cell density in the subsequent scale up stage. It is expected that most processes would avoid these problems in the process development of cell banking and seed expansion. Finally in the production process, changes in a simple process parameter such as pH would be expected to affect lactate production, which subsequently would affect base addition and osmolality as discussed earlier. Some cell culture production processes are sensitive to osmolality [31,33]. Osmolality induced by base addition is then further complicated by the type of base used. Many cell culture processes utilize carbonate as compared with a hydroxide source to adjust pH. While hydroxide contributes a single cation equimolar for each contribution of base, carbonate used as base contributes two cations as well as the corresponding bicarbonate equilibrium to the total osmolality. Besides the osmolality effect, there will be an additional increase in dissolved carbon dioxide that may also affect growth, viability, metabolism [57,58] and PQ [59]. From a related standpoint, physical differences between production lines in a manufacturing facility may lead to unintended biological variation. Consequently validation of similar production lines to demonstrate the equivalency is important such as recently reported by Minow and coworkers [60]. Some differences between production lines can be determined through extensive computational fluid dynamics and detailed physical characterization of bioreactors, and minimizing the differences between bioreactors can aid with scalability and transfer of production lines [60–65]. These differences may lead to changes in dissolved carbon dioxide, for example, which in turn would affect pH and have the aforementioned effects on culture. Yet the combination of all of these sources of biological variation, from vial to production, would be expected to yield variation in peak cell density and potentially the process would yield different Q_p and PQ.

An example process

With a consistent vial to production process in place, the inherent cell culture process variability can be examined within the context of the analytical capabilities. In Figure 2, normalized process data are summarized from 10 production experiments performed in bench-scale bioreactors with example time course data of viable cell density, viability, pH, pCO_2, osmolality, glucose, lactate, ammonia and titer. The average data set is presented along with the time courses representing the average plus two standard deviations (SDs; referred to as the 'high process') and the average minus two SDs (referred to as the 'low process'). Using two SDs captures 95% of the expected performance based on the inherent reproducibility possible of the entire system. This example data set represents an instance of a consistent process. With an established reproducible process, true process variation becomes easier to diagnose and ultimately identifies the source of the problem. In this case study, the cell growth and productivity fall within the aforementioned range for a nonvariable process of less than 15% variation. Despite the consistency of this process, one of the largest relative differences in these data at harvest between high and low processes of the nine parameters considered is the glucose concentration. To determine the source of the discrepancies between different control experiments, one must look at the

sources of the data. As the analytical variation from measuring the glucose concentration is expected to be in the order of only 10%, this is only a fraction of the variation observed. Therefore a majority of the changes in measured glucose concentration must be directly process related.

A logical conclusion would be that differential growth would yield the different harvest glucose concentrations. When analyzing the data presented in Figure 2, the variation in cell growth depicted could be entirely captured by variation in cell counters as the data set was generated over time in a laboratory using multiple automated cell counters. Despite some variability in glucose, any actual differences in cell growth from the high process to the low process cannot be discerned by using an automated cell counter. All samples from individual experiments utilizing automated cell counters ideally should be analyzed on the same cell counter for consistency. This was the practice in generating the data in Figure 2. As a general rule, data sets comparing growth measured on different cell counters should be analyzed with the inherent difficulty in cell counting accuracy in mind. While it is possible that glucose consumption differentiated as a result of the different cell densities achieved, this cannot be conclusively determined.

With respect to the other process parameters presented, variation in glucose represents approximately 50% of the osmolarity differences between the high and low processes presented in Figure 2. Approximately 25% of the osmolality difference is explained by variation in base addition resulting in a concomitant sodium and osmolality increase (data not shown). As osmometers are very accurate, the other divergence

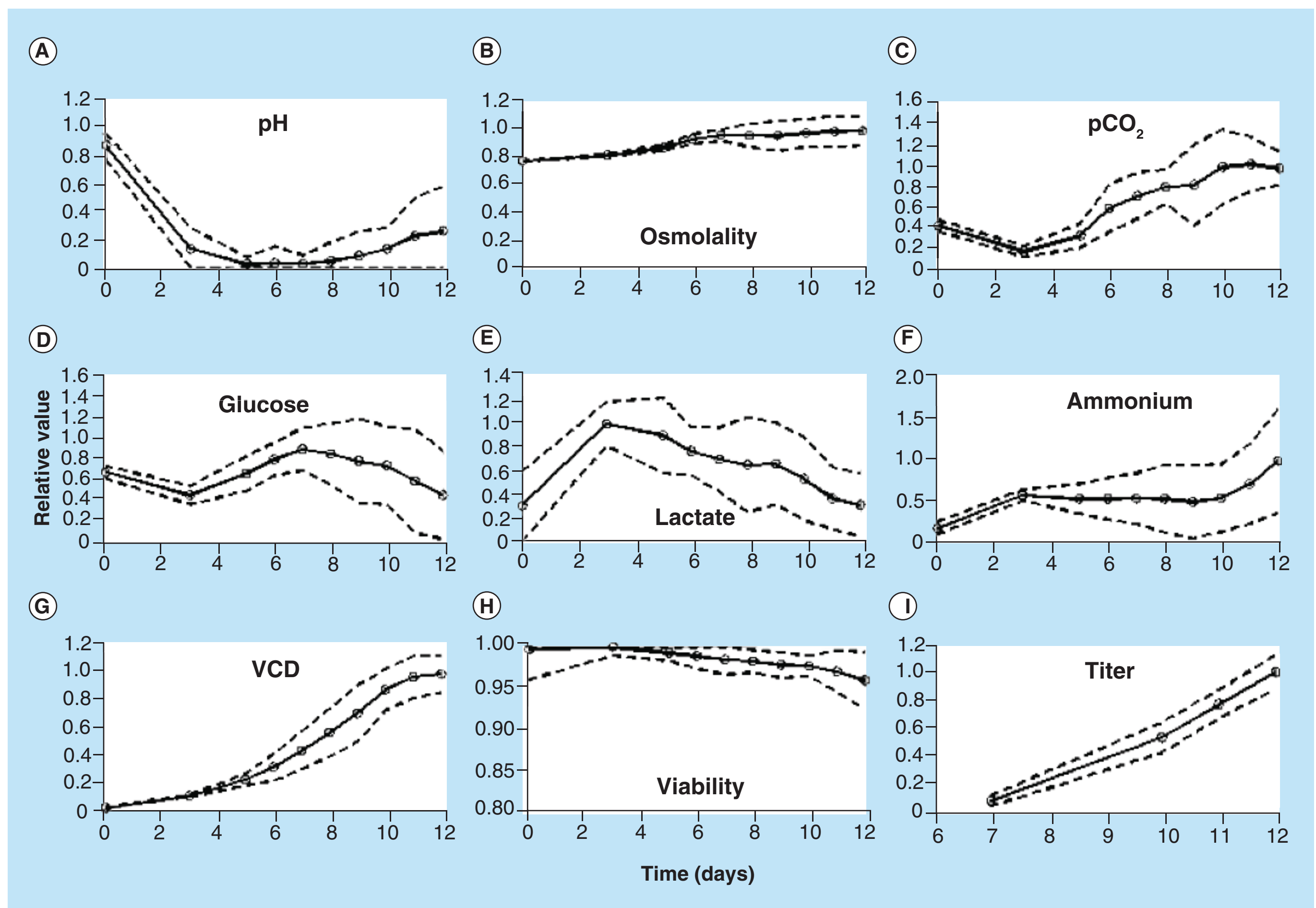

Figure 2. Relative time-course profiles for cell culture-process parameters. The average process (n = 10) is represented by open circles, the average +2 standard deviations and the average -2 standard deviations are represented by dashed lines. Process parameters presented are pH, osmolality, pCO_2, glucose, lactate, ammonium, VCD, viability, and titer. In the case of pH, physical limits of the system are imposed on the plot depicting the average -2 standard deviation data so as not to depict a pH outside of the controlled pH. A similar physical limitation is imposed on viability data for the plot of average + 2 standard deviation to prevent viability being reported above 100%.

pCO_2: Partial pressure of carbon dioxide; VCD: Viable cell density.

must be due to analytes other than glucose and base. The discrepancy in lactate and ammonium measurements are not large individually but could be used to explain the total variation observed in osmolality when combined with glucose and base consumption. Despite these variations, the titer and thus the volumetric productivity of the culture show minor differences.

Overall, the process depicted in Figure 2 was robust with respect to cell growth to the degree that can be discerned by the measurement technique. The question then remains what would cause the changes resulting in the high process glucose concentration as compared with the low process glucose concentration. When comparing the differences at inoculation to the analytical capability to measure changes, the variation in process inputs are within the error of the measurements. The process inputs depicted are therefore not in question. The major source of variation then would be expected to be biological in nature, which may include cell growth differences. Such an example is a useful tool against which to compare more extreme variation. In a condition where the process was varying to a concerning degree, a targeted question could then be posed as to what may induce changes in glucose consumption rate. As cells are sensitive to changes in media formulation, the sections that follow will focus on how mammalian cells respond to the extracellular environment, how the media may be different than the intended formulation and how inherent variability in some media formulations affects the robust performance of the process with respect to productivity and PQ.

Bioreactor composition

One seemingly innocuous decision is the choice of bioreactor composition for the production process. Historically large-scale cell culture processes were cultivated in stainless-steel bioreactors, with stainless steel expected to not affect the cell culture. However, stainless steel is known to leach metals including iron, nickel and chromium [66–69]. Even extreme measures, such as extensive washing regimens, to eliminate trace metals are not sufficient as metals will still leach from equipment [70]. While most of the literature in this area pertains to cytocompatability for medical devices and final product formulations, cell culture production processes are also neutral pH systems, in biologically relevant buffers, with similar metal leaching profiles. The leaching rates of metals shown in these studies would result in generally low concentrations in cell culture media. However, given that metals are frequently present in trace amounts in cell culture media, the process must be designed to be robust to these potential contributions. In addition, variation in metal content of cell culture media has been shown to impact PQ and productivity. These effects are discussed later in the cell culture media composition section of this review.

Over the last decade, there has been a significant expansion of the disposable bioreactor market with an expected market share of 20% over the next few years [71]. Disposable bioreactors have been shown to be scalable up to 1000 l with similar performance to traditional bioreactor systems at up to 15,000 l scale [72]. Unfortunately disposable bioreactors have also been the source of several problems. Hammond and coworkers identified bis(2,4-di-tert-butylphenyl)phosphate as a toxic leachable compound from a disposable cell culture bag [73] which caused poor cell growth. Others have developed a generic growth test methodology prior to manufacturing use of disposable systems [74,75] and even screening up to 13 disposable bag types and 8 vendors [76,77]. Growth inhibition again by a toxic, leaching compound from the cell culture or media container has to be considered and eliminated. In addition, cholesterol-dependent cell lines required novel solutions in order to permit cultivation in disposable bioreactors as the cholesterol has been shown to interact with the plastic resulting in cell growth inhibition [78,79]. While the bioreactor composition may initially seem innocuous, this is not a decision that can be ignored when designing a robust process. Differential concentration in metals or toxic organic compounds varying across a manufacturing campaign clearly has the potential to induce variability in process performance. As the stainless-steel bioreactor is not the only potential source for metal contribution to the system, further discussion on metals is reserved for the section on cell culture media composition.

Variability in cell growth may result in PQ changes

A common focus for industrial application is identifying how a process variation leads to a measured PQ change. In this section, we aim to simplify the process variation to a consideration of either overgrowth or undergrowth. Beginning with the overgrowth condition, it is easy to consider that increased cell mass will occur concomitantly with decreased extracellular nutrients due to increased consumption of nutrients to create the cells. Complete consumption of amino acids may lead to cessation of growth even when the amino acids are nonessential [80]. Cessation of growth due to overgrowth early in a process may lead to a steep decrease in cell viability, which can impact PQ due to increased sialidase activity for example [81]. Later in the culture, the decreased peak cell density will ulti-

mately lead to an overfeeding condition, potentially to high lactate levels [45], but certainly to high osmolality. As discussed earlier, high osmolality [31–33] can affect glycosylation. Alternatively overgrowth can lead to consumption of a particular amino acid and subsequently the cell may substitute a different amino acid for the desired amino acid in a protein [82,83]. Sequence variants may have an effect on efficacy as even single amino acid changes can impact antibody–antigen interactions [84]. Besides amino acids, glucose concentration will inevitably be decreased by increased growth early in the process. Decreased glucose concentrations may lead to changes in sialylation and high mannose species [85]. Complete glucose deprivation for extended periods has been shown to result in 45% nonglycosylated antibody [86]. Furthermore some processes are tailored to achieve optimized levels of galactosylation through feeding specific components to specified concentrations [10,32,87]. Increased cellular consumption of these nutrients may decrease the effectiveness of these strategies.

From the perspective of undergrowth in cell culture, overfeeding certainly will occur, and nutrient depletion will not be a concern. However, excessive extracellular nutrition will be a concern. Cysteine concentration has been shown to directly impact trisulfide content of monoclonal antibodies [88] and accumulation of cysteine due to undergrowth leads to increased trisulfides. Trisulfide bonds are disulfide bond with an extra sulfur introduced in the linkage. Trisulfides have been identified on a wide variety of antibodies [89–91]. Glycation, which is a nonenzymatic chemical reaction between glucose and the antibody, is a PQ concern [92,93] and is caused when glucose accumulates extracellularly. Excessive nutrients also lead directly to high osmolality and the subsequent effect on glycosylation. High osmolality can induce apoptosis [58], decrease growth [57] and increase sialidase levels [81]. To combat these issues, Yuk and coworkers have specifically developed a strategy around avoiding glycation by controlling glucose feeding [94]. Anti-apoptotic genes including Bcl-x_L have also been a major focus for research in order to limit this pathway's effect on productivity and PQ [3,95–97].

Cell culture media composition

Cell culture media composition is a critical factor for cell culture process productivity and PQ. Historically processes were designed based on the productivity enhancing properties of hydrolysates or sera [98,99]. Hydrolysates and sera are inherently complex components and therefore are not consistent. To handle the variability, screening lots becomes a necessary exercise [100–102] and screening different hydrolysate sources has been shown to effect productivity and PQ [103]. Recent work has investigated the nature of the components enhancing productivity and growth [104,105]. Sera have largely been eliminated from current process development workflows as an attempt to limit exposure to adventitious agents. But both hydrolysates and sera should be eliminated to enhance process consistency.

Due to this raw material variability, the industry has shifted to chemically defined media. Several reports have demonstrated direct improvements in productivity by optimizing media [2–4,106]. Consequently consistency in cell culture media is critical, as the media were specifically designed for the productivity and PQ which result from that chemical composition. Despite removing lot-to-lot variability by shifting to a defined media formulation, there have been several reports of issues. Sodium carbonate was identified as an unexpected source of copper that affected productivity [107]. In the context of modern chemically defined processes which achieve very high cell mass and productivity, trace elements become even more important. Unintended metal contributions from raw material components potentially can also affect PQ. Changes in the copper concentration have been shown to affect basic variants and aggregation [108,109]. As another potential example, an increase in manganese concentration in media has been shown to affect glycosylation site occupancy and galactosylation [10,11,32,87,110]. A chemically defined process would be designed with a specific manganese concentration as the intended concentration and expected PQ. In these examples then, a shift in manganese or copper concentration may result in PQ out of specifications, similar to the example of unexpected productivity from the unintended copper. A critical aspect of the robust performance anticipated from chemically defined media is the routine production of the same desired media formulation every time. When ordering a proprietary media formulation from two different vendors, there are reports of media arriving as different colored powder clearly demonstrating that the chemical composition was not identical from the two suppliers [111]. Various factors including the blending and heat generated during the media powder milling process have been implicated in chemically defined media variability [112]. The solubility, in fact, of individual amino acids has been shown to not be a constant from vendor to vendor or lot to lot [113].

In addition, chemically defined media has been linked directly to the PQ attribute of drug substance color. Recent reports have linked iron and vitamins [114,115], and even a vitamin not natively in the cell culture process [116], to terminal drug substance color. In

the latter case, the vitamin identified to cause the pink drug substance color was a light degradation product of another vitamin. Light degradation of media can also affect cell culture performance and leads to decreased cell growth via a mechanism likely related to degraded riboflavin and tryptophan [117]. As a result of these challenges, LC/MS [117] and fluorescent approaches [118] have been developed to monitor the degradation products.

Eliminating variability to establish process consistency

Cell culture media are a source of process variability, whether from complex or chemically defined media. Establishing firm control over the quality of the cell culture media to be used in an industrial process therefore is paramount to achieve process consistency. Table 1 summarizes a wide variety of currently published methodologies used to analyze cell culture media. To a large degree, most of these methods involve a screening approach, either from an analytical chemistry or cell culture perspective. There has been a strong shift toward this screening approach, easily observable in the literature, with an emphasis on spectroscopic methods [100,101,112,119–126]. Spectroscopic methods enable detection of new or unexpected components as well as the possibility of simply detecting difficult to quantitate differences. As a result, it has been possible to detect differences in hydrolysates and ultimately correlate to performance [100,101,120,124]. An alternative approach is to actively screen these complex components with a use test and cell culture [102]. Both approaches can be summarized as identifying the problem in a process input and preventing the problem from entering the bioreactor. In addition, both approaches require implementation of an active monitoring program for potential problematic media ingredients. Such a program requires the identification of the root cause of the variability *a priori*. Screening out problems ahead of time is a direct solution to establish process consistency and should lead to the elimination of some variability.

However, not all problems may be due to identified media concerns or even to the media itself. The next logical step is to institute superior control over the bioreactor process step itself. In this vein of thinking, spectroscopic methods have recently become very popular as ways to monitor cell culture [127–139] as replacements for more traditional methods of monitoring [85,140–143]. The methods are targeted at measuring a wide variety of common cell culture parameters, including glucose, biomass and even off-gas analysis, with many of these analyses capable of providing continuous at-line or online monitoring. Table 2 summarizes the bioreactor monitoring techniques as well as whether the techniques were used as part of a feedback control methodology. Of these technologies, off-gas analysis used for controlling feeding regimes in a feedback loop is the best established [144,145]. Similar approaches have been used more recently using an open-loop controller and a predictive model, measuring oxygen consumption and maintaining glutamine at appropriate levels in order to ensure process consistency [146,147]. Focused-beam reflectance measurement was utilized to measure biomass accumulation online [148]. An at-line HPLC was utilized to measure charge variants every few hours in one of the few direct measurements of PQ [149].

To date, most of the reports demonstrate pure monitoring approaches, as only a few methods have already been implemented as feedback control strategies shown to improve processes. As pH is routinely measured online as part of industrial cell culture processes, utilizing pH as an approach to control glucose concentration was very effective at maintaining low lactate concentrations [42]. Alternatively, glutamine was con-

Table 1. Summary of raw material monitoring tools and approaches.

Methodology	Problem identified	Control approach	Ref.
Biological	Lot-to-lot variability in hydrolysates	Cell culture use test	[101–103]
Fluorescence excitation–emission matrix spectroscopy	Light degraded media	Screen media	[121]
Fluorescence spectroscopy	Variation in basal medium powder and hydrolysates	Screen media	[100]
Liquid chromatography/mass spectrometry	Light degraded media	Screen media	[117]
Near-infrared spectroscopy	Variation in basal medium powder	Screen media	[112,120,126]
Nuclear magnetic resonance	Lot-to-lot variability in hydrolysates	Screen media	[124]
Solubility	Variation in intra- and inter-vendor lots	Screen suppliers	[113]

Table 2. Summary of bioreactor monitoring tools and parameters measured.

Methodology	Feedback control implemented	Parameters measured	Ref.
At-line HPLC	No	Charge variants	[149]
Automated flow cytometry	Yes	VCD, TCD, viability, cell size	[151]
	No	VCD, TCD, viability, cell size, cell cycle	[150]
Dielectric spectroscopy	Yes	VCD	[1]
	No	VCD, cell size	[129,133]
Focused beam reflectance measurement	No	Biomass concentration	[148]
Glucose fed based on pH	Yes	pH	[42]
Near-infrared spectroscopy	No	Glucose, lactate, glutamine, ammonium, osmolality, VCD, PCV	[128,131,132,134]
Off-gas	Yes	Oxygen, carbon dioxide	[144–147]
	No	Oxygen, carbon dioxide	[135,139]
Online metabolite sampling	Yes	Glucose, lactate, ammonium, glutamate, glutamine	[1,85,142]
	No	Glucose, lactate, VCD, viability	[140,141]
Raman spectroscopy	Yes	Glucose, lactate, glutamine, ammonium, VCD	[153]
	No	Glucose, lactate, glutamine, glutamate, ammonium, VCD, TCD	[127,137,138]

PCV: Packed cell volume; TCD: Total cell density; VCD: Viable cell density.

trolled through online measurement via a YSI 2700 biochemical analyzer, resulting in decreased ammonia and increased cell growth [85]. If glutamine was allowed to deplete to less than 0.1 mM, high mannose species were increased and sialylation decreased, which shows the criticality of the control method in this process. Tsang and coworkers developed an optimized feedback control strategy based on online sampling every 3 h and measurement of various metabolites leading to improved productivities [142]. A similar approach was presented by Lu and colleagues with glucose controlled at a targeted level as well as the demonstration of feedback control based upon online capacitance in parallel [1]. While the report shows that glucose monitoring was the superior target, the metabolite monitoring occurs every few hours. Automated flow cytometry has been implemented to monitor viable cell density, cell size and cell cycle [150] and also to control fed batch operation [151].

Spectroscopic methods for measuring metabolites hold additional promise over these automatically sampled systems requiring withdrawal of culture from the bioreactor. The spectroscopic methods are noninvasive and capable of measuring far more analytes at a far more frequent rate. Continuous monitoring of cell culture greatly increases the knowledge base as to the direct metabolic kinetics as compared with traditional daily sampling. Daily sampling can completely obfuscate meaningful dynamics. The shift toward continuous monitoring of cell culture is a step in the right direction. Implementation of such technologies in manufacturing settings requires the combined applied knowledge base of analytical chemistry, biochemistry and engineering. However, advanced monitoring still is only a step. To truly establish consistent processes, these spectroscopic methods could be used in feedback loops to control the process at a desired set point [152]. Some initial work has recently demonstrated that glucose could be controlled in a feedback loop using a nonlinear model predictive controller and Raman spectroscopy as the measurement technique [153], which demonstrates the future potential applications.

Conclusion & future perspective

With all of the positive attributes of systems such as near-infrared and Raman spectroscopy, several of the reports discuss relatively large measurement errors of over 10% of the targeted analyte which may make a continuous feedback control strategy challenging. Yet it is clear that these spectroscopic methods provide a great deal of data more than a traditional autosampler

connected to a metabolite analyzer could provide. The power of more frequent online sensing combined with further improvements in chemometrics and the appropriately designed control strategy should ultimately lead to better controlled cell culture processes. From a simplified perspective, it is quite clear that pH control is best achieved through online pH measurement as compared with offline, infrequent pH sampling. The parallel to that simplified example in most current industrial processes is the fixed nutrient feeding schedules that cannot account for variations in performance as only offline, infrequent cell growth and analyte data are available. Whereas the current standard is fixed nutrient feeding schedules, the future contains dynamic feeding schedules determined by advanced control loops dictated by spectroscopic sensors contained within the bioreactor. This idealized future assumes that feedback control using spectroscopic techniques will eventually reach the level of true process analytical technology. To achieve this goal, the chemometric field must continue to advance such that individual analyte measurements become more reliable and the controllers that depend on chemometric measurements can be more effective. For proper implementation of such advanced control loops, soft sensors are likely to be involved. Luttman and colleagues have recently described the current status of soft sensors and made additional recommendations including the expectation that these sensors simplify industrial standard operating procedures [154].

Once the industry fully understands the competing factors that result in the variable measurements, the path forward will clearly be to harness that information for true process control generating consistent batches in terms of yield and PQ. Processes designed with these capabilities in mind are speculated to be dynamic as the process would be adjusted potentially to account for cell growth, metabolism and potentially even PQ. Such a vision will only come to fruition from an extensive amount of process development knowledge. Implementing that process knowledge into a control strategy, combined with detailed nearly continuous or continuous online trending of both cells and metabolism, is the desired future state. Processes which include induction steps critical to productivity or PQ would in particular obtain additional control through the use of a dynamic process strategy. The induction could be specifically targeted at the appropriate time based on the increased trending capabilities. When that process is validated at a manufacturing site, then the manufacturer will have complete control over both PQ and Q_p in those batches.

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Executive summary

Background

- Mammalian cell culture processes may be variable in both productivity (Q_p) and product quality (PQ).
- Control of the supply chain and the safety of the product require process consistency.
- Cellular metabolism can affect PQ as changes in metabolic waste products such as lactate and ammonia can ultimately result in altered PQ.

Defining process variability

- Acceptable process variation is defined as less than 15% in a measured cell culture parameter.
- There are external sources of variation, such as analytical technique variation and instrument-to-instrument variation, which may contribute substantially to the total measured cell culture variation in a process.

Sources of variation

- The physical bioreactor composition may affect process outcomes, whether due to the contribution of metals leaching from stainless steel vessels or toxic organic molecules leaching from disposable single-use vessels.
- Product modifications may change from a variety of sources, including amino acid and glucose depletion as well as amino acid and glucose accumulation.
- Shifting to chemically defined media from complex media additives has not completely eliminated sources of variation in cell culture. Variation in drug substance color has been a recent point of interest in the literature.

Eliminating variability to establish process consistency

- Spectroscopic tools have been applied to screen raw materials as well as to monitor cell culture performance. These tools have been used to even predict cell culture performance from raw material screens.
- Spectroscopic tools can monitor the bioreactor more frequently than automated sampling devices to more traditional analyzers. However, feedback control in order to truly generate process consistency using spectroscopic tools still requires additional investment in order to achieve such a goal.

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