Introduction

The cells used in cell culture processes are all derived from animals. Their genetic and epigenetic control circuits and their physiology have evolved to carry out the physiological functions of the tissue from which they were derived, not to sustain themselves and proliferate in *in vitro* culture. To gain insight into how to grow them well, it is instructive to first see how they are nurtured in their native environment. The human body is, in a way, the best bioreactor. More accurately, it is a reaction system consisting of many bioreactors. Figure 9.1a depicts a few organs in our body that perform complementary functions to sustain our body over a long period of time. The body operates as a batch system, with periodic input of nutrients and output of waste streams from excretions. An adult human body consists of about 3.7×10^{13} cells.[1] A modern bioreactor can reach ~10^{10} cell/L at the end of a batch culture. In terms of quantity of cells, a human body is equivalent to an industrial bioreactor of ~400 L in size. In general, cells in a bioprocess reactor consume many times the daily recommended intake of both carbohydrates and amino acids for an adult. Of course, the comparison is simplistic since cells in the reactor are growing while most cells in an adult are not dividing and may require a smaller amount of nutrients. Nevertheless, our body is certainly more efficient in nutrient utilization. What are the major differences between growing cells in a culture and growing cells in a body?

The composition of the food we take in daily usually does not match the exact needs of our body. Furthermore, both the intake of nutrients and our bodies' needs for different activities change over time. Cell metabolism generates waste metabolites, some of which may become toxic

if allowed to accumulate to high levels. Our bodies cope with such imbalances by using the liver as a metabolic factory to balance chemical compositions in the circulating blood and detoxify inhibitory metabolites by degrading them or converting them to less inhibitory compounds (Figure 9.1b). The liver also serves as a reservoir of glucose to maintain glucose levels in a range of ~4 to 7 mM in the blood. The kidney then purges the waste metabolite from the body and the lungs remove carbon dioxide from the bloodstream. These balancing actions by multiple organs keep the nutrient and metabolite levels in the bloodstream in check. With these organs keeping nutrient and metabolite levels in a relatively narrow range, cells evolved to thrive only in relatively invariant chemical environments, making them rather intolerant of large deviations from their normal chemical environments. This intolerance has narrowed the allowable range of nutrient and metabolite levels in bioreactors for optimal cell growth.

A drastic difference between a bioreactor and a human body is the absence of a metabolite-balancing mechanism. The accumulation of lactate, ammonium, and other metabolites of amino acid degradation in a bioreactor inhibits growth. In cell culture bioreactors, the nutrient is often added initially or fed periodically. Upon feeding, the nutrients accumulate to high levels and then decrease to low levels due to consumption. Bioreactor operation is largely based on the strategy of sustaining an

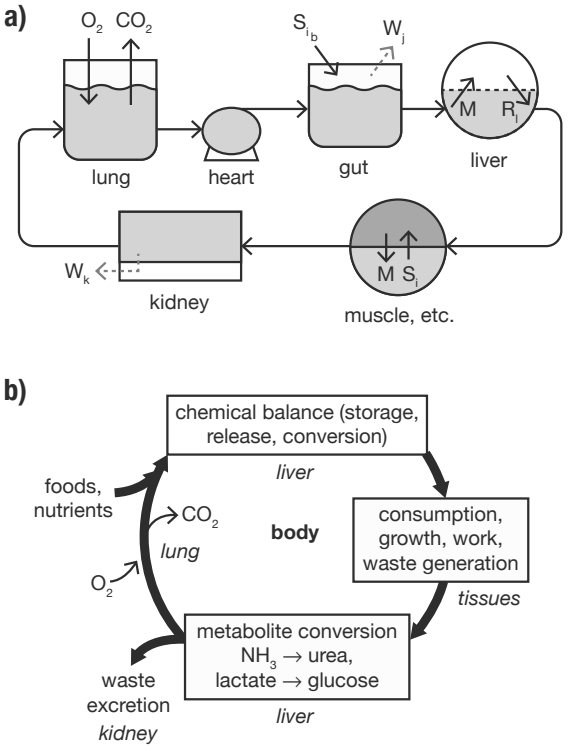

Figure 9.1. Organs as a bioreaction system. It consists of oxygen supply, carbon dioxide removal, a mixing provider, a chemical conversion factory, and a waste disposal mechanism. In the gut, nutrients (S) from food are absorbed into blood circulation, leaving some waste (W) for excretion. In the liver, nutrients and metabolites from different organs are processed to maintain a balanced state and some metabolites are chemically transformed (R) into waste. In muscle and other tissue, nutrients are consumed to generate energy for performing work and to grow more biomass, all the while producing metabolites. In the kidney, many metabolites (including R from liver metabolism) are excreted. Finally, CO_2 from various tissues is exhaled and fresh oxygen is supplied to the blood, where it recirculates back to different organs via blood pumping.

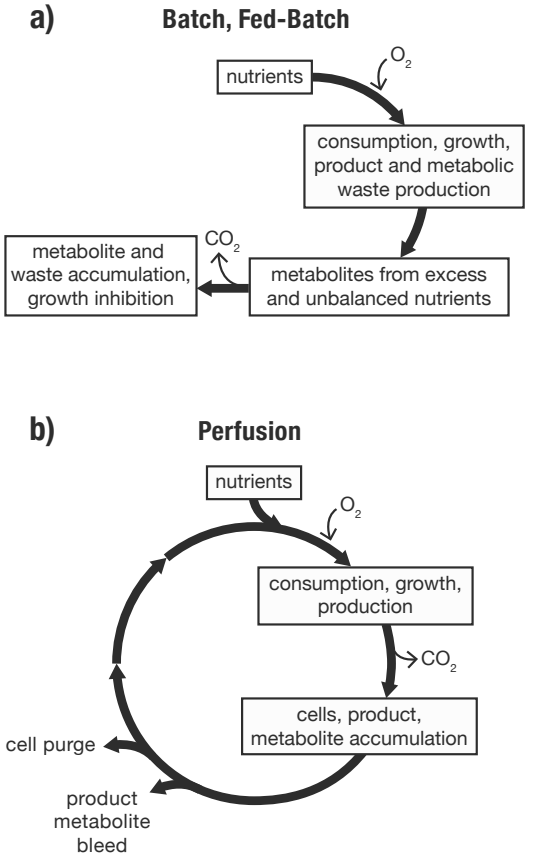

Figure 9.2. Cell culture processes lacking the chemical conversion and balancing capability of the organs' system. Balancing nutrient supply can minimize the accumulation of metabolites in batch/fed-batch cultures and alleviate the need for a high perfusion rate.

optimal chemical environment profile in order to maximize productivity and robustly deliver a high-quality product.

The initial level of nutrients in cell culture processes is typically higher than that seen in our bodily fluids. Even that level, however, is insufficient to grow cells to the density needed for industrial production. Hence, most industrial cell culture production processes are operated in a fed-batch mode. The main limiting factor for productivity in a fed-batch operation is the accumulation of metabolites and culmination of other growth inhibitory factors. A major cause of the metabolite accumulation is the unbalanced supply of nutrients (Figure 9.2a). A key factor in increasing the productivity of a fed-batch culture process is providing a balanced chemical environment that minimizes the production of metabolites and prolongs the time it takes for them to accumulate to inhibitory levels.

The operation of a bioreactor is generally classified as batch or continuous mode. In a continuous process, the feed is continuously being introduced to the reactor and the product stream is continuously being withdrawn. In a batch process, the medium, including all the nutrients (except oxygen), is

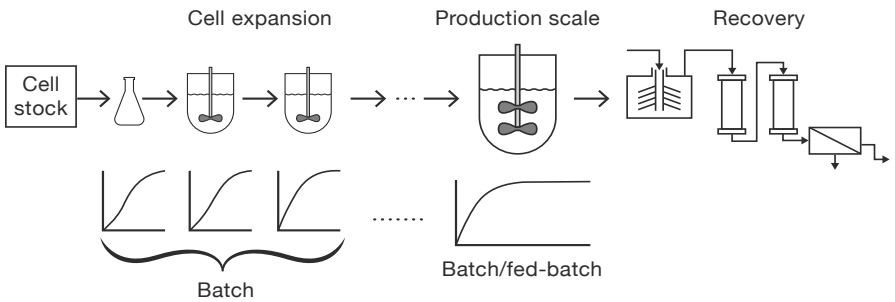

Figure 9.3. A typical cell culture process for the production of biologics combining batch and fed-batch cultures followed by downstream processing.

added at the beginning to support cell growth and production until the end of cultivation. A variant of batch process is the fed-batch process, in which additional nutrients are added during the cultivation period to sustain the growth and production longer than that of a batch culture. The vast majority of cell culture manufacturing processes employ batch culture for cell expansion and fed-batch culture in the production-scale reactor (Figure 9.3). The series of batch cultures for cell expansion is often referred to as a seed train. At the termination of a fed-batch reactor, the cell viability may be low and the product is recovered in downstream operations. Continuous culture is not commonly practiced in the cultivation of mammalian cells unless it is in conjunction with cell recycling. This process allows for continuous discharge of metabolites and product, thus alleviating the growth inhibition caused by metabolite accumulation and prolonging the culture (Figure 9.2b). The different types of continuous cultures will be discussed in the next chapter.

Batch Cultures

Batch processes are simple and widely used. In fact, batch process culture is used much more frequently than fed-batch, though in smaller scales in seed trains. Batch culture is used for the production of viruses for vaccines and gene therapy applications as well as in cell production for immunotherapy and other therapies.

Kinetic description of batch culture

The growth behavior of a cell line in a batch culture can be described by setting up the material balance equations of the reactor on the cells, the key nutrients and metabolites, and possibly the osmolality, which all influence the growth rate (Panel 9.1). Note that material balance is performed on the quantity of the chemical species to be balanced, not the concentration of the species, as the volume of the system being balanced may change, thus rendering a balance on concentration invalid. In the case of a batch culture, the volume is constant (Eq. 9-1). We therefore eliminate the volume from the equations, thereby allowing balances on cells (x), substrate (i.e., nutrients; s), and the inhibitory metabolite (I) to be based on concentrations (Eqs. 9-2, 9-3, and 9-4). By incorporating a description of the dependence of the specific growth rate on the level of substrate and inhibitor (e.g., the Monod type of relationship (Eq. 9-2) and tying the production of the inhibitor to the consumption of the substrate by a stoichiometric coefficient, the kinetic model can be used to generate a growth curve for a batch culture (Figure 9.4). In most cases, the effect of inhibitory metabolite accumulation has a greater effect on cell growth than the reduction of substrate.

Panel 9.1. Material Balance for a Batch Bioreactor

$$\frac{dVx}{dt} = V\frac{dx}{dt} = V\mu x \qquad \textbf{(Eq. 9-1)}$$

$$\frac{dx}{dt} = \mu x = \frac{\mu_{\max}}{K_s + s} \cdot \frac{1}{1 + \frac{I^2}{K_I}} x \qquad \textbf{(Eq. 9-2)}$$

$$\frac{ds}{dt} = -q_s x \qquad \textbf{(Eq. 9-3)}$$

$$\frac{dI}{dt} = \alpha_{I/s} q_s x \qquad \textbf{(Eq. 9-4)}$$

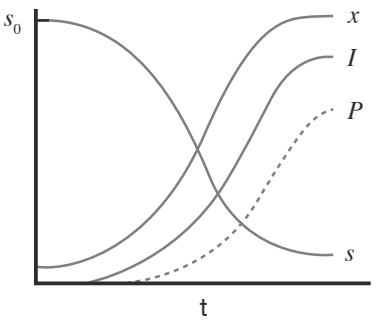

Figure 9.4. The kinetics of cell growth, substrate, product, and metabolites in a batch culture.

Fed-Batch Cultures

Batch cell cultures are limited by the low cell and product concentrations achievable. To achieve a high cell and product concentration, the initial nutrient concentration in the medium must be high. Excessively high nutrient levels and the accompanying high osmolality causes growth inhibition; hence the use of fed-batch processes.

Types of Fed-Batch Cultures

Intermittent harvest

The simplest form of fed-batch culture involves intermittent harvest. At a late exponential growth stage of the culture, a portion of the cells and product are harvested, and the culture is replenished with fresh medium. This avoids metabolite inhibition of cell growth and replenishes nutrients for continued cell growth. This process is typically repeated several times before the culture is terminated (Figure 9.5). This simple strategy is commonplace for the production of viral vaccines produced by persistent infection, as it allows for an extended production period. It is also used in roller bottle processes with adherent cells. By intermittent harvesting, turn-around time involved in cleaning up and restarting the process is saved. Furthermore, after replenishing the medium

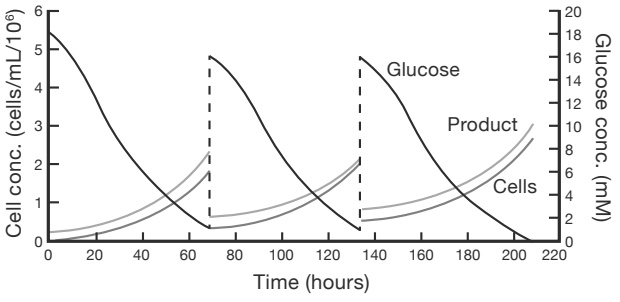

Figure 9.5. An intermittent fed-batch culture.

the culture reaches peak cell concentration faster than if the culture had been started all over. In some cases, this type of fed-batch culture is also used to supply inoculum to start the seed train for production for a period of time. Cell products, such as those to be used for cell therapy, cannot be subjected to highly stressed conditions such as in fed-batch culture and are therefore typically produced in batch cultures or intermittent fed-batch cultures.

Fed-batch culture with fortified feed

For production of recombinant proteins, a fed-batch culture is initiated by partially filling up the bioreactor with the startup medium (approximately 70% of the reactor capacity, or at a level sufficient to allow the impeller to be submerged) (Figure 9.6a). Nutrients, usually in a more concentrated solution (fortified feed) than in the startup medium, are added during the cultivation. The concentrated medium is added either continuously or intermittently. The additional nutrient supply prolongs the growth period and increases the cell concentration. It also extends the period of product synthesis and results in a substantially higher product titer. It is common practice to decrease the operating temperature after a period of rapid growth in fed-batch culture. The temperature shift reduces the growth and metabolic rates, extending the production period and increasing the product titer. During the late stage of a fed-batch culture, the osmolality in the medium is high due to the addition of fortified medium (which is commonly prepared with an additional alkali or acidic solution to facilitate the solution of solid nutrients) and a pH-neutralizing base. The osmotic stress also enhances the secretion of proteins.[2, 3] The practice of this type of fed-batch culture led to the enhancement of the productivity of cell culture processes that we saw in the decade around the turn of the century.

The increased cell concentration and prolonged process in a fed-batch culture amplifies the adverse effect of metabolite accumulation. In addition to the usual inhibitors such as lactate and ammonium, the degradation products of other compounds, for example aromatic and aliphatic amino acids, may also reach inhibitory concentrations. The high osmolality and accumulation of metabolites and reactive oxygen species all contribute to the eventual loss of viability and productivity of the culture. The resulting cell death and possibly cell lysis likely causes the release of cellular enzymes such as proteases and sialidase. The release of cellular enzymes has been attributed to the increased occurrence of structural and glycosylation variants at the end stage of fed-batch culture. Thus, most fed-batch processes do not operate until the viability becomes low.

As discussed in Chapter 3, at a stationary phase of culture cells may change from a high glucose flux/high lactate production state to a low

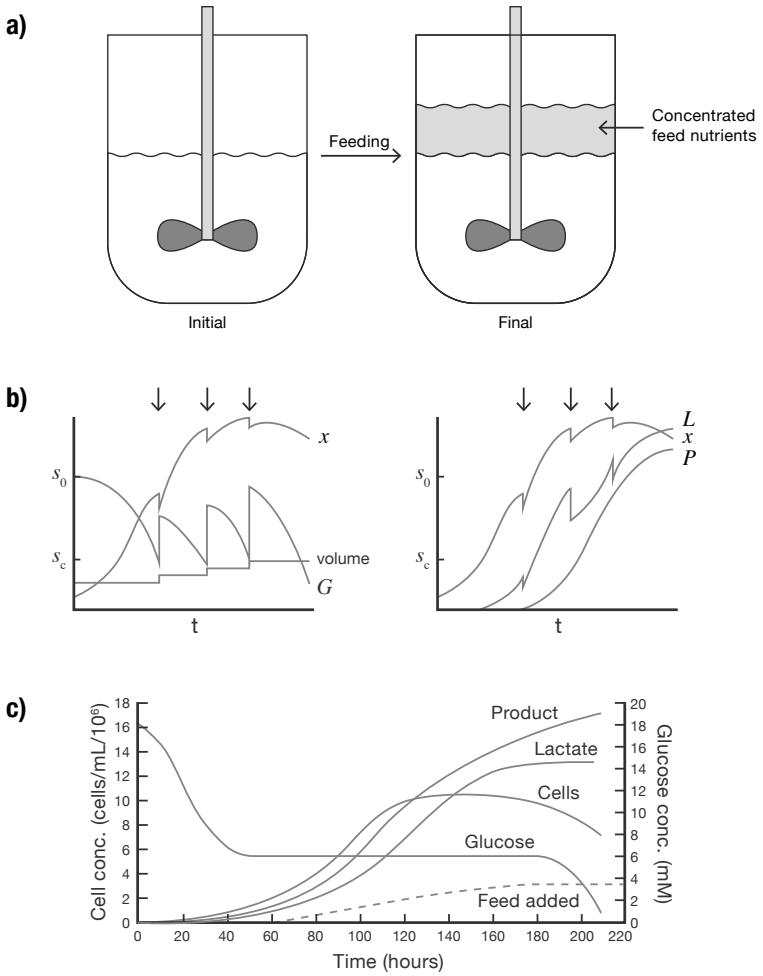

Figure 9.6. (a) Fed-batch culture with increasing volume during cell cultivation by (b) periodic feeding as indicated by ↓ and the step change in the concentrations, or (c) continuous feeding.

glucose flux/lactate consumption state. The metabolic shift is generally accompanied by a decreasing level of lactate, increasing pH, and a slowed increase of osmolality. In this period one also often observes a higher productivity.

Kinetic Description of Fed-Batch Culture

The most important design parameter of a fed-batch process is the feeding scheme, both in terms of the composition of the feed and the scheduling of feed addition. The main culprit of inhibitory metabolite accumulation arises from over or imbalanced supplies of nutrients. Balancing the supply of nutrients reduces metabolite accumulation and

increases cell concentration and productivity. A kinetic model describing the state of the culture will greatly facilitate the formulation of a feeding scheme.

In a fed-batch culture, the volume of the culture is changing over time (Eq. 9-5, Panel 9.2). The volume change is the result of feeding at a rate of F. Implicitly we assume that the density of the culture is constant, so the balance can be done on the culture's volume instead of its total mass. The differential equations for the material balance will include the culture volume, which considers the total amount in the reactor, instead of balancing on the concentration (Eqs. 9-6, 9-7, and 9-8). The biomass balance equation is the same as that for batch culture. For nutrients, we use subscript i to denote different medium components that are consumed by cells. If a component of the medium is not consumed, then the consumption term (the second term) on the right-hand side of Eq. 9-7 is zero. We assume a single feed is used to supply all nutrients. The production of metabolites (I) is related to a reference substrate (for example, glucose or glutamine) with stoichiometric ratio α between I and s_i (Eq. 9-8).

Panel 9.2. Material Balance for a Fed-Batch Bioreactor

$$\frac{dV}{dt} = F(t) \qquad \textbf{(Eq. 9-5)}$$

$$\frac{dVx}{dt} = \mu x V \qquad \textbf{(Eq. 9-6)}$$

$$\frac{dVs_i}{dt} = Fs_{f_i} - q_{s_i} x V \qquad \textbf{(Eq. 9-7)}$$

$$\frac{dVI}{dt} = \alpha_{I/s_i} q_{s_i} x V \qquad \textbf{(Eq. 9-8)}$$

We consider two cases, one feeding continuously and the other intermittently. In the first case, one needs to determine the feeding rate as a function of time and feed concentration. In the latter, one determines the amount of feed to be added so that by the next feeding time the concentration can be maintained above a set level.

For the first case, we aim to maintain the substrate concentration constant. We thus set Eq. 9-7 equal to 0 and solve for the feeding rate. By the product rule (Eq. 9-8) and rearrangement, Eq. 9-7 becomes Eq. 9-11 (Panel 9.3). The feeding rate is dependent on the feed concentration s_{f_i}. Using a constant feed concentration, the flow rate increases with increasing cell concentration as cells grow.

For the second case, the balance between two discrete time points, t_1 and t_2 (Eqs. 9-12 and 9-13, Panel 9.4) is used to determine the amount of nutrients that need to be fed. In the equation, we retain the symbol of feed as F, even though in practice it is added at only t_1. We then integrate from t_1 to t_2 and use M to denote the amount of feed added at t_1 (Eq. 9-14). By adding M amount, we aim to supply the substrate so that its concentration does not exceed or fall below a certain range. M is thus dependent on the amount of substrate consumed in the interval between feedings (Eq. 9-16). After the addition of the feed, the substrate concentration increases (Eq. 9-17). The higher the nutrient consumption in the

Panel 9.3. Continuous Feeding of a Fed-Batch Culture

From Eq. 9-7, to maintain constant s (i.e., $\frac{ds}{dt} = 0$)

$$V\frac{ds_i}{dt} + s_i\frac{dV}{dt} = 0 + s_iF = Fs_{f_i} - q_{s_i}xV \qquad \textbf{(Eq. 9-9)}$$

$$F\left(s_{f_i} - s_i\right) = q_{s_i}xV \qquad \textbf{(Eq. 9-10)}$$

$$F = \frac{q_{s_i}xV}{s_{f_i} - s_i} \qquad \textbf{(Eq. 9-11)}$$

Panel 9.4. Step (Intermittent) Feeding

$$\frac{\Delta\left(Vs_i\right)}{\Delta t} = Fs_{f_i} - q_{s_i}xV \qquad \textbf{(Eq. 9-12)}$$

$$\Delta Vs_i = Fs_{f_i}\Delta t - q_{s_i}xV\Delta t \qquad \textbf{(Eq. 9-13)}$$

Integrate between t_1 and t_2, feed is added in the amount of $F\Delta t$ at t_1. $F = 0$ at all other times. We use M to denote the total amount of feed added at t (this keeps the units correct in the balance equation).

$$M = F\Delta t \qquad \textbf{(Eq. 9-14)}$$

$$Vs_i\Big|_{t_1} - Vs_i\Big|_{t_2} = Ms_{f_i} - \int_{t_1}^{t_2} q_{s_i}xV\,dt \qquad \textbf{(Eq. 9-15)}$$

Can determine M to be added at t_1

$$M = \left(Vs_i\Big|_{t_1} - Vs_i\Big|_{t_2} + q_{s_i}xV\Delta t\right)/s_{f_i} \qquad \textbf{(Eq. 9-16)}$$

Eq. 9-16 is used to iterate M and Δt to find an operating combination.

After the addition of feed, the substrate concentration increases to s'_{i,t_1}

$$s'_{i,t_1} = \frac{s_{i,t_1}V_{t_1} + Fs_{f_i}\Delta t}{V_{t_1} + F\Delta t} \qquad \textbf{(Eq. 9-17)}$$

interval of feeding is (i.e., the higher the cell concentration or the longer the interval), the higher the concentration after the feeding will be. With a shorter interval (i.e., more frequent feeding), the amount of substrate cells consume is smaller, thus allowing a lower level of substrate concentration upon feed addition. A scenario of a fed-batch culture profile is shown in Figure 9.6.

Fed-Batch with Metabolic State Manipulation

With the realization that metabolite accumulation limits cell concentration and productivity in fed-batch culture came several strategies designed to alleviate the problem. The first approach induces a metabolic shift from a high glucose flux state to a low one during the rapid growth stage in order to eliminate lactate accumulation. This is accomplished by stoichiometric feeding of glucose, glutamine, and other amino acids using the strategy described in the preceding section. By keeping the concentration of glucose at a consistently low level, glycolysis is shifted to a low flux state characterized by the reduced production of lactate and ammonium (Figure 9.7).[4] The glucose concentration is in the vicinity of K_M for glucose transporter GLUT1 (see Chapter 3). The strategy thus requires continuous nutrient feeding to maintain glucose in a narrow range; too high will lead to a return to a high flux state, too low may cause glucose depletion and apoptosis. In another approach, the controlled feeding is only implemented when rapid cell growth is over. The feeding can be linked to pH control, as either lactate production or consumption can lead to control actions for pH fluctuation.[5]

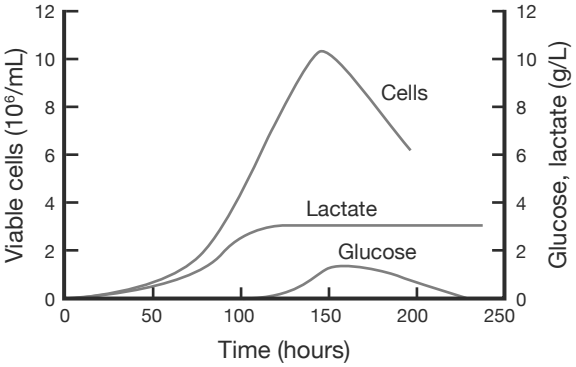

Figure 9.7. A fed-batch culture with continuous feeding to control nutrient levels and shift metabolism. Keeping glucose at low levels reduces glucose consumption and lactate formation. Reducing both glucose and glutamine levels reduces lactate and ammonium accumulation.

Stoichiometric Feeding

Critical to a successful fed-batch process is the formulation of feed media and a feeding strategy. Much of the inhibitory metabolite accumulation in fed-batch culture is due to an unnecessarily high level of nutrients caused by unbalanced supply. A sound feeding strategy is to provide a balanced nutrient medium in order to minimize the accumulation of inhibitory metabolites.

Under balanced growth conditions, cells consume various nutrients and produce different metabolites in some proportionality. These proportionalities are called stoichiometric ratios. The idea of stoichiometric feeding is that if all nutrients are consumed in relative proportions, one can feed all nutrients according to the stoichiometric ratios. If one supplies a balanced amount for one nutrient and keeps all the other nutrients at their correct stoichiometric ratios, then every nutrient will be supplied in a balanced amount and maintained within an optimal range. The stoichiometric ratio can thus be used as a basis for designing feed media.

Medium for Stoichiometric Feeding

A well-formulated feed media seeks to keep all nutrients at the same proportion as their consumption (i.e., at their stoichiometric ratios). The stoichiometric ratio is determined experimentally using the same cell line under relevant cultivation conditions. Typically, one medium component is chosen as a reference nutrient. The consumption rates of all other nutrients are related to the reference nutrient by its stoichiometric ratio. Common choices for reference nutrients are glucose, glutamine, oxygen, and lactate, as they are consumed or produced in larger quantities and are relatively easy to measure. As discussed in Chapter 5, the stoichiometric ratio is easily calculated from the cumulative consumption of nutrients and the production of metabolites. The cumulative consumption of a nutrient is plotted against that of the reference nutrient from the same culture. The slope on the plot gives its stoichiometric ratio.

Stoichiometric ratios are not necessarily constant. Cells may consume different nutrients in different proportions in different culture stages. The feed media composition may need to be adjusted according to known changes of stoichiometric ratios under different culture conditions or in different growth stages. The development of a stoichiometric feed medium takes a couple iterations. A medium with the first composition is tested and analyzed to derive a new set of stoichiometric ratios for a new feed medium, which is then tested in turn. It usually takes about three iterations for an optimal medium to emerge. In addition to the feeding medium, another variable is the optimal level a given nutrient ought to maintain in culture. Most essential amino acids need to be maintained above a critical level. Even some non-essential amino acids should be kept above certain levels because their deficiency may lead to amino acid misincorporation in the product or other physiological effects.

Once the composition is determined, it is ideal to make a highly concentrated feed medium to increase the amount of nutrients that can be delivered using a fixed volume of feed. However, this is limited by the solubility and kinetics of solution of the various components. Many components are slow to dissolve. A highly concentrated medium may also be

prone to precipitation in storage. Typically, the concentration of nutrients in the feed medium is about 10 to 15 times that in basal medium.

Unconsumed Medium Components

Some medium components are not consumed or are consumed at such a minute rate that their concentration change in a typical culture is not detectable. Some examples include inorganic ions like sodium, calcium, and sulfate. These hardly-consumed medium components may not need to be added to the feed, but because the culture volume increases upon feeding they will become diluted. Consequently, feeding of these unconsumed components may be necessary if their dilution affects growth or product synthesis. When included in the feed, they are at basal medium levels so that they are not diluted by the expanding volume (Panel 9.5).

Panel 9.5. Feed Medium

Consumed Nutrients

- Present in the medium proportional to their stoichiometric consumption

Unconsumed

- Many components are consumed at very low levels, such that their concentration in the culture supernatant hardly changes
- They can be omitted from the feed or be present at basal medium levels to minimize dilution caused by culture volume expansion
- Feeding may cause an osmolality increase
- The "consumption" of hydrolysates, growth factors, etc., may not be easily quantified

Some ions, such as magnesium (complexed with ATP), phosphate (as free phosphate or in nucleotides), and potassium, are present at a much higher concentration intracellularly than in the medium. At high cell concentrations, the amount of these ions taken up by the cells may become significant. Therefore, it is often necessary to compensate for their consumption by supplying them in the feed medium.

In addition to basal medium components, protein hydrolysates, serum, insulin, transferrin, vitamins, and lipid additives are also used in the start-up medium. These additives may supply minute nutrients that are not identified and measured. In the absence of a reliable measurement of consumption rate for those additives, one has to rely on an order-of-magnitude estimate of the upper and lower limits of their consumption rate. The feeding rate of those additives can then be chosen to maintain their concentrations above a minimum threshold and below a maximum tolerable limit which is experimentally determined.

Another point of consideration in feed medium design is its effect on culture osmolality. In a basal medium, various salts and buffers contribute to the bulk of osmolality. The feed medium, in principle, should maintain osmolality balance in a culture. Immediately after feeding, the osmolality may increase, but, because the feed is stoichiometrically balanced, after the nutrients are consumed the osmolality should return to the original value unless additional salts are present in the feed. In

practice, the feed typically has a high osmolality and high salt concentration in addition to the nutrients. Because some amino acids dissolve very slowly, an acid or base is often added during feed medium preparation to accelerate dissolution and then neutralized after solution. The added acid or base increases the salt content in the medium. Higher osmolality in the production stage has been shown to enhance productivity. However, overly high osmolality reduces cell viability. Keeping osmolality under an upper bound is thus part of the design consideration.

Delivery of Feed Medium

Intermittent Feeding

How the feed medium is delivered to a culture may affect the performance of a fed-batch process. In most manufacturing settings, the feed medium is added intermittently. The time point and amount of feeding is either prescribed in the operating protocol or based on the measurement of a variable (e.g., glucose concentration) that is to be kept within a certain range. Since the addition of feed increases osmolality, the first addition is performed after the initial rapid growth stage. Cells will then continue to grow at a slower rate. As shown in the balance equations, for the period between two consecutive feeding times, t_1 and t_2 (Eq. 9-12 to Eq. 9-15), the amount of nutrient delivered at t_1 (Ms_{if}) should be sufficient to supply the demand ($q_{si}xV\Delta t$) and sustain s_i above the set point level. At higher cell concentrations, more feed medium is added. That inevitably leads to a higher level of post-feed nutrients ($s'_{i,\,t1}$) which may cause excessively high levels of nutrients and magnify inhibitory metabolite accumulation. Alternatively, a smaller amount of feed can be added more frequently to keep the post-feed nutrient level within an acceptable range.

Continuous Feeding

Continuous feeding minimizes the medium composition's tendency to swing from high to low levels periodically as caused by intermittent feeding. It also offers the possibility of manipulating cell metabolism, for example to implement a more robust switch from a high flux glucose metabolism to lactate consumption in the late stage. As the concentration increases, the feeding rate has to increase to meet the increased demand. This requires frequent, ideally on-line, measurement of a process variable that can be used to adjust the feeding rate.

Feeding by Direct Measurement of Nutrient Consumption

Since nutrient feeding is based on stoichiometric balance, direct measurement of the concentration of the reference substrate is the most straightforward way to determine the amount of nutrient that has been consumed and how much feed should be added to balance the consumption. By measuring the reference nutrient concentration on-line, feed can be added in real time to maintain the nutrient at a constant level. Glucose and glutamine are the two nutrients most commonly measured and used as controls because their concentrations can be determined fairly rapidly in laboratories. Direct measurement of these compounds on-line requires using an auto-sampling device that is connected to a measurement device.

Proportional Feeding with Cell Concentration

A strategy of continuous feeding is to increase the flow rate proportional to cell concentration. Various laser turbidity probes can be used to determine the cell concentration in culture. Capacitance probes are increasingly being used because they measure total viable cell volume (i.e., viable cell concentrations and cell sizes) instead of the total cell concentration (both viable and dead cells) given by optical probes. Feeding with a simple proportionality to cell concentration assumes that all the other contributors to the cells' nutrient demands as shown in Eq. 9-11 are constant. This is an approximation that works well for a period of time. If the nutrient concentration does not need to be controlled in a narrow range, this method is easy to set up if an on-line cell concentration measurement is available.

Stoichiometric Feeding with Base Addition

A widely-used nutrient feeding strategy in microbial fermentation links nutrient feeding to acid or base addition for pH control. When the uptake of a nutrient or the production of a key metabolite is associated with the release or dissipation of H^+, a stoichiometric relationship can be established between them and the amount of acid or based added to neutralize pH can be used to determine the amount of nutrient consumed. In cell cultures, a large fraction of the glucose consumed is converted to lactic acid, and in the late stationary phase lactate may be consumed. To maintain pH, one mole of base or acid is added to compensate for the H^+ excreted or taken up with lactate through MCT transporters. From the amount of base/acid added, the rate of lactic acid production can be calculated. That in turn is used to feed the stoichiometrically balanced medium and maintain nutrient levels. In other words, the method uses the amount of base or acid added to maintain pH to determine the $q_{si}xV$ term in Eq. 9-11. It is simple and easy to implement. However, its

response is sluggish because of the buffer capacity of the medium. The dissolved CO_2 level in the medium varies over a wide range throughout the culture time. Hence, the estimated lactate production is subject to errors. However, most manufacturing processes do not control nutrient levels in a narrow range, and the method is adequate even for reducing glucose flux.

Stoichiometric Feeding with Oxygen Uptake Rate (OUR)

The oxygen uptake rate (OUR) is a very sensitive method for assessing cellular metabolic activity that dictates the demand for nutrients and the feeding rate. A typical dissolved oxygen sensor can detect a change of 5% saturation with air at 1atm, or about 0.01 mmol/L. That is much more sensitive than typical laboratory measurement for glucose (about 0.3 mmol/L). In principle, pH measurement can detect very small differences of H^+ concentration, but the large buffer capacity in a biological fluid decreases its sensitivity for detection. OUR measurements, unlike pH, are not masked by buffers in the medium. OUR measurement is also relatively easy to implement on-line as discussed in Chapter 11.

With its high sensitivity, small changes of OUR can be confidently detected on-line and in real time, thereby providing an immediate indication of changes in metabolic rate. Its measurement is indicative of the overall metabolic activity, a combined effect of the specific rate and the cell concentration as opposed to the cell concentration measured using a capacitance probe. Through the established stoichiometric ratio, OUR measurement can be used to determine the feeding rate (Eq. 9-18, Panel 9.6). The metabolic rate change in cell culture processes is relatively slow. In most cases, intermittent determination of OUR in intervals of tens of minutes is sufficient to detect metabolic rate changes and adjust the feeding rate.

Panel 9.6. Stoichiometric Feeding by OUR

- OUR is a sensitive method for quantifying cells' metabolic load
  - Detect Δc ~0.02 mmol/L accurately (for glucose, Δc for detection ~ 0.3 mmol/L)

$$F = \frac{\alpha_{s/o}(\mathrm{OUR})V}{s_{f_i} - s_i} = \frac{\left(\alpha_{s/o} q_{o_2} x V\right)}{s_{f_i} - s_i} \qquad \textbf{(Eq. 9-18)}$$

Product Quality in Fed-Batch Culture

The quality of the therapeutic protein produced in culture is the ultimate concern of biomanufacturing. Important quality attributes include the amino acid sequence, chemical modifications of the protein, and glycosylation. A number of intrinsic characteristics of fed-batch culture give its product a potentially wider range of heterogeneity compared to batch or continuous culture.

Quality Variation in Different Growth Stages

Seeking to enhance productivity, many fed-batch cultures employ culture conditions that incur high stress. The osmolality often increases from the 300 mOsm that is optimal for growth to over 400 mOsm in the final stage of production, as it increases product titer. Some fed-batch culture processes reduce the cultivation temperature once the rapid cell growth stage is over. A fed-batch culture thus has very distinct stages: the early stage, in which the chemical environment is optimal for growth; the middle stage, in which the environment is highly stressed; and the late stage, in which cells are non-proliferating due to the addition of a fortified medium and an accumulation of metabolites. During the rapid growth stage, cells are invariably in a high glucose flux and high lactate producing state. In many cases, a shift to low glucose flux and lactate consumption is seen in the late stage of fed-batch culture, as discussed in Chapter 3. Cells are at very different metabolic states in those stages and are also likely to have very different intracellular concentrations and many metabolic intermediates that may affect post-translational modification of proteins. Cells at these distinct stages with different chemical environments, temperatures, growth rates, and metabolic states, may produce products with different quality attributes.[6]

Overfeeding or undersupplying of nutrients affects product quality. Since the effect of errors is cumulative in a culture, the effects of over- or under-feeding is more profound in the late stage of culture. The depletion of a particular amino acid may cause amino acid misincorporation in the protein product. In place of the correct amino acid that is in short supply, the wrong amino acid is incorporated in the protein. Oversupply and consumption of aromatic and aliphatic amino acids increases the production of reactive oxygen species and other metabolites that may increase the number of protein structural variants.

Typically, the quality of the product produced is assessed by examining the quality attribute of the purified protein harvested at the end of the culture, which is the accumulation of all protein molecules produced from the beginning of the culture to the end. If we could isolate only the product produced during a given period of time in the culture, one would be able to assess how the quality of the product varies with the culture's stages. However, such information is rarely collected. If it becomes available, it will facilitate the development of a control strategy for product quality.

Quality and product residence time

Product molecules produced at different points of time in a fed-batch culture spend different durations of time in the reactor before being harvested, i.e., the product molecules have different "ages." As illustrated in Figure 9.8, product produced earlier spends more time in the culture, and the amount of product produced differs among the stages. Cells in

different stages are at different metabolic states and may produce product of varying quality. In some cases, the culture environment causes the secreted product to undergo changes; product secreted at different times, therefore, will spend different durations in the culture and have a different probability of being modified. For example, sometimes the culture conditions involve a very high glucose concentration (15 g/L or 83 mM) (recall that the physiological range is 0.7–1.2 g/L) that increases the likelihood of glycation in the protein product. A prolonged culture under stressed conditions increases cell death and the release of cellular materials, including sialidase, which removes sialic acid from glycan and

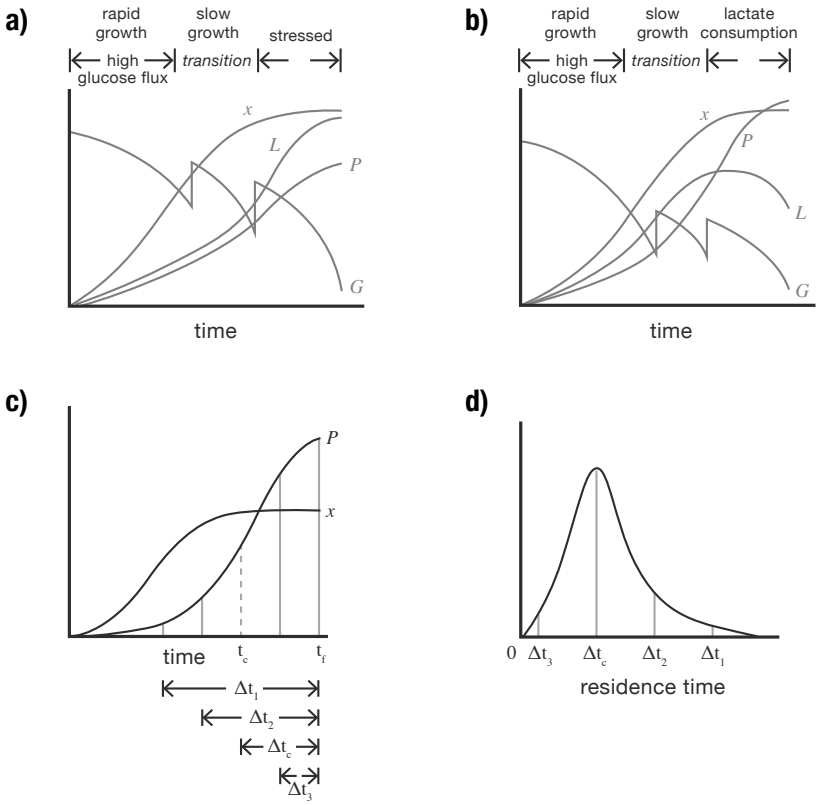

Figure 9.8. (a, b) Product proteins are produced at different stages of a culture with different physiological states. Different cultures may have different metabolic behavior; one (a) produces lactate until the end, while the other (b) switches to lactate consumption. The different physiological states may secrete proteins of different qualities. (c) The quality of the final protein product is the average of all the molecules produced at different times across the culture. t_c is the time point when half of the product molecules have been produced, and t_f is the end of culture. (d) Protein molecules secreted earlier have a longer residence time (or age) (Δt). Since they spend different amounts of time exposed to the culture fluid, they are subject to varying degrees of modification.

gives rise to asialilated glycans.[7] Various proteases and lytic enzymes released from dead cells may alter product protein quality. To minimize cell-death-related protein quality deterioration, some researchers maintain a long, slow growth phase and avoid long periods of low viability.

Quality and Culture Reproducibility

A fed-batch culture involves more process variables and is subject to wider variability than a batch culture. The presence of distinct culture stages and the disparate residence time among products, bring additional heterogeneity to the product quality. If the cells' physiological conditions change in different cultures, detectable alterations to the quality attributes of the product produced in different runs may be seen.[8] When such variations occur, the investigation of the cause may need to examine products produced at different stages of the culture to pinpoint the cause.

In some cases, different process runs under the same manufacturing conditions, with the same cell line, and at the same manufacturing plant can behave quite differently. For example, some runs switch from a high glucose flux state to a low flux and lactate consumption state, while others don't. These two types of culture would have very different metabolic states. The glycan produced under those two conditions appears to bear differences. Under different metabolic states, the pool size of nucleotide sugars may differ, thus causing glycoform inconsistency among different runs. A feeding strategy that can better guide cell metabolism to a consistent state will allow for a more robust production of a quality product.

Concluding Remarks

Fed-batch culture is the prevailing mode of cell culture in the final production of manufacturing recombinant proteins. It extends the cell growth and production periods, reaches high cell concentrations, and is operationally relatively simple. The design of the feed medium and the selection of the feeding strategy strongly influence the productivity of fed-batch culture. Compared to batch and continuous culture operations, cells in fed-batch cultures are subjected to wide variations in their chemical environment and are potentially physiologically different during different stages of their culture as well as in different runs. Enhanced process knowledge and better control of the chemical environment will enhance the consistency of product quality.