Introduction

In a biomanufacturing process, cells take up nutrients which generate energy and are converted to more cells, metabolites, and product. For many microbial biochemicals, the cost of those raw materials constitutes a major portion of the total production cost. It is therefore important to maximize the efficiency of converting the raw materials to the product. Most cell culture products are not raw-material intensive. However, efficient nutrient utilization is still important. Over-abundance and over-consumption of nutrients leads to inhibitory metabolite accumulation, which affects productivity and even product quality.

Cells convert materials through their biochemical reaction network. Depending on the chemical environment, nutrients are taken through different routes as they are converted to cells, metabolites, and product. These routes have different material utilization efficiencies and, more importantly, different effects on overall process productivity. During conversion, the principle of material balance cannot be violated. Using the material balance principle to establish the stoichiometric relationship between inputs and process outputs is the first step for process design and enhancement.

When optimizing a process, in addition to the information on the stoichiometric relationships between materials, it is important to know how to appropriate those materials over time and how the outcome variables will behave in response. Quantitative descriptions of how the important material variables (e.g., cell biomass, nutrients, metabolites) change with other process variables and time are key tools when

developing a plan to guide the process along a desired path. This chapter discusses the stoichiometry and kinetics of cell growth in a bioprocess.

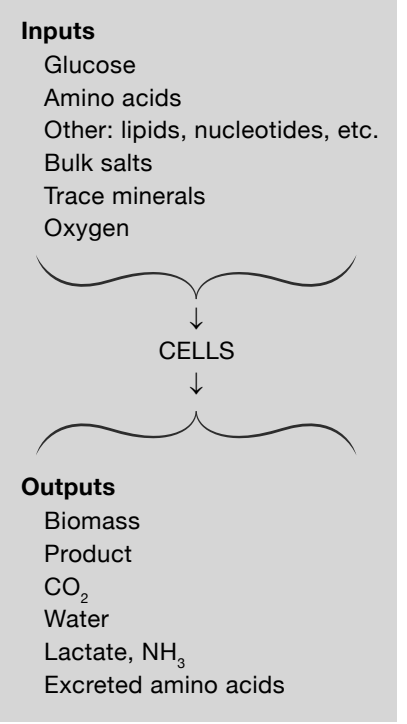
Material Conversion in Cell Culture

Cells obtain their material resources from their medium (Panel 5.1), including water, which is the most abundant component in a cell. Additionally, cells consume oxygen so they can derive energy from the nutrients they consume. The three nutrients consumed in the greatest quantity by most cells in culture are oxygen, glucose, and glutamine. Some adapted or engineered cells do not require glutamine. Inside the cell, the material inputs are converted in the cell's biochemical reaction network to metabolites in the course of generating energy and synthesizing cell mass and product. To generate energy, glucose is converted to lactate, CO_2, and H_2O through glycolysis, the TCA cycle, and the pentose phosphate pathway. Glutamine is deaminated, releasing NH_3, before its carbon skeleton enters the TCA cycle through α-ketoglutarate for energy metabolism. The major metabolites generated from energy metabolism are thus lactate, NH_3, CO_2, and H_2O. The amino group from the metabolized glutamine and other amino acids may also be exported as non-essential amino acids (e.g., alanine, asparagine, proline) in addition to being excreted as NH_3. The energy metabolism generates ATP, NADH, and NADPH to meet the energetic needs of many biosynthetic reactions.

In the course of metabolic material conversion, many reaction intermediates are generated, most of which continue to be catabolized or are used in biosynthesis to make other cellular components. Some will be secreted as metabolites. The line between intermediates is a blurred one. Many reaction intermediates, such as components of the TCA cycle, are also secreted, albeit in small proportions to the amount synthesized. For example, virtually all of the lactate synthesized during cell growth is secreted as metabolites, while only a small portion of the TCA cycle intermediate citrate may be secreted under some culture conditions.

From a material balance perspective, we can treat growing cells as a system on which the material balance is to be performed. The inputs include all materials that are taken up by cells, i.e., the consumed nutrients (say, over a time period or, if one

Panel 5.1. Inputs and Outputs of Cell Growth Processes



can measure it so quickly, at an instant of time). The outputs include the new biomass that is synthesized and the metabolites, including lactate, NH_3, CO_2, and H_2O that are secreted. The balance thus focuses on the exchange of materials between the system (cells) and its environment. In the following sections, we will discuss the major items of inputs and outputs in a cell culture system. Material balance is also performed on the intracellular bioreactor network based on the material exchange with the environment. The analysis, referred to as metabolic flux analysis, will be discussed in Appendix A.

Cell Biomass

Before material balance on cell growth can be performed, one has to determine the material content of the cell. The size of a cell varies widely from microorganisms to those of mammals. Order of magnitude-wise, the dry biomass of an average cell of bacteria, yeast, and animal cells is 10^{-12}, 10^{-11}, and 5 x 10^{-10} g per cell, respectively (Table 5.1). The most abundant chemical species in a cell is water, accounting for up to 90% of the volume of plant cells, ~80% of animal cells (Table 5.2), and 70% of bacterial cells. Since cell cultivation is carried out in aqueous environments and the amount of water taken up by cells during growth is difficult to measure, the material balance on cell culture is typically performed only on "dry" matter, thereby excluding water.

Table 5.1. Typical Dry Weight of Cells

Type	*Dry weight*
Bacterial	10^{-12} g/cell
Yeast	10^{-11} g/cell
Average Animal Cell	3–6 x 10^{-10} g/cell

Macroscopically, cells are made of a few classes of macromolecules (proteins, carbohydrates, DNA, and RNA) or macromolecular assemblies (primarily lipids). These organic matters constitute the vast majority of the dry mass in a cell. Protein molecules constitute the largest portion among them, providing the machinery for DNA, RNA, and protein synthesis. Protein molecules also serve as the structural components of the cell and execute all of the catalytic, transport, and communication functions. The lipid content of an animal cell is higher than in a bacterium. The abundance of organelles and the presence of a lipid bilayer membrane in an animal cell contributes to their higher numbers.

Table 5.2. Average Composition of an Animal Cell

	pg/cell	*Range*	*%*	*% of dry biomass*
Wet weight	3500	3000–8000		
Dry weight	600	300–1200		
Protein	250	200–300	10–15	~50–70
Carbohydrate	150	40–200	~1–5	~5
Lipid	120	100–200	~1–2	~5
DNA	10	8–17	~0.3	~2
RNA	25	20–40	~0.7	~4
Water			55–80	
Volume	4 x 10^{-9} cm^3			

Intracellular carbohydrates exist as oligosaccharides on many proteins and lipids. Carbohydrate

also exists as five carbon carbohydrates (ribose and deoxyribose) in RNA, DNA, and nucleotides (e.g., ATP, GTP). Only a small fraction exists in a free (or phosphorylated) form. The cellular content of carbohydrates is harder to estimate because most carbohydrates are conjugated to lipids, proteins, or nucleosides.

Variation in cell size

Cells isolated from different tissues or of different differentiation types vary in size (Table 5.3, Panel 5.2). The volume of a typical mammalian cell is in the order of a few picoliters (about 1,000 times larger than a bacterium). The average diameter of a population of cells in culture ranges from 10 to 20 μm. Cells of different differentiation types that were isolated from the same tissue often differ in size. For example, megakaryocytes are much larger than T lymphocytes and natural killer cells. Even hepatocytes isolated from different regions (zones) of the liver differ in size. Pluripotent stem cells and other more primitive stem cells are fairly small. Their nucleus spans more than 70% of the cell diameter and their cytoplasm is relatively small. Liver cells and antibody-secreting plasma cells are at the other end of the cell size spectrum, containing a significant amount of cytoplasm for protein secretion.

Even cells in a homogeneous population of the same cell type or cell line have different sizes. Cells are concurrently undergoing different stages of the cell cycle; those immediately before mitosis are twice as large as those after cell division. Like many other properties, such as DNA content per cell, the cell size of a population distributes over a range with more than a 2-fold difference between the highest and lowest subpopulations. For aneuploid cells, the distribution of size is typically greater than for normal diploid cells. Some aneuploid cell lines have a wide distribution of chromosome numbers, and some even have a tetraploid karyotype. Those cells are larger than diploid cells.

The distribution of cell size changes with the growth stage. Rapidly growing and quiescent cells may have different sizes. Furthermore, in a culture, cells that lose viability often become visibly smaller, as measured by flow cytometry.

Table 5.3. Size of Animal Cells

Cell type	*Volume (μm^3)*	*Diameter (μm)*
Hybridomas	900–4000	12–20
Endothelial cells	1400–2500	14–17
Chinese hamster ovary cells (suspension)	1200–1800	~14
Human foreskin fibroblasts (FS-4)	3000–6000	18–22

Panel 5.2. Size of Cells in Culture

- Cell volume distributes over a range
- Dead cells are often smaller
- Cell size varies with culture stage

Cell number is traditionally used for the quantification of cell concentration, even though it does not sufficiently capture the metabolic and production capacity of the culture since cell size may change during the culture and among different cultures. It is instructive to remember that the volume of a sphere (which is a reasonable approximation of a cell) is proportional to its diameter raised to the third power. Therefore, cells that are twice as large in diameter are eight times larger in cell volume. In addition to cell size variation, the density, or the water content, of a cell may also vary in different stages of a culture. In a fed-batch culture, the osmolality increases over culture time, possibly causing the density of cellular content to vary. Thus, under some conditions, such as investigating cell growth or productivity change, it is necessary to quantify cell size distribution and cellular density in addition to cell number.

Amino acid composition of cellular proteins

Microbial and plant cells often grow on simple carbon sources supplemented with an inorganic nitrogen source, such as ammonium or urea. These cells convert inorganic nitrogen to all 20 natural amino acids that are used to make proteins. Animal cells lack the capability to make 11 to 12 of those 20 natural amino acids. These essential amino acids must be supplied for animal cell culture to enable them to grow and make products.

Table 5.4. Approximate Amino Acid Composition of Cells and IgG

	Cell composition mean (%)	*Standard deviation*	*IgG composition*
Ala	9.03	0.32	5.31
Arg	4.74	0.32	2.43
Asn	10.08	0.59	3.49
Asp			3.95
Cys	0.26	0.04	2.43
Gln	12.62	0.63	5.01
Glu			5.16
Gly	9.14	0.57	6.98
His	2.22	0.07	1.67
Ile	5.73	0.35	2.43
Leu	9.00	0.68	6.83
Lys	6.85	0.49	6.98
Met	2.27	0.14	1.37
Phe	3.73	0.31	3.49
Pro	5.51	0.58	7.13
Ser	6.19	0.16	12.90
Thr	5.42	0.22	7.74
Tyr	2.73	0.14	4.10
Val	6.54	0.27	9.10

The protein content and composition of cells change under different growth conditions; however, they are seldom measured. Table 5.4 lists the approximate amino acid composition of a cell and IgG. Given target levels of cell biomass and product to be produced, a stoichiometric amount of all essential amino acids must be supplied. In addition to essential amino acids, non-essential amino acids are usually also supplied in a cell culture medium.

Intracellular fluid

Water constitutes 70–80% of total cell volume. The soluble low molecular components (excluding polymeric materials and lipid membrane assemblies) in water also make up a large fraction of the biomass. The intracellular fluid contains electrolytes, carbohydrates, amino acids, metabolic intermediates, nucleotides (ATP, ADP, etc.), and many other components. The vast majority

of intracellular amino acids reside in cellular proteins and only a small fraction exist as free amino acids in intracellular fluid. Most amino acids are present at the 0.05–0.5 mM range in intracellular fluid. Their normal concentration is sufficient to sustain a high fidelity in charging their corresponding tRNA.

Typical concentrations of amino acids and other major soluble components in intracellular fluids are listed in Table 5.5 and Table 5.6, respectively. The intracellular concentration of amino acids can vary widely among different cell lines and under different growth stages and culture conditions. Also shown in Table 5.6 are the typical concentrations of solutes in the cell's natural environment (i.e., the interstitial fluid). Potassium, magnesium, and phosphate are present at high concentrations (millimolar range). A large fraction of cellular Mg^{2+} is associated with ATP, which is typically present at the 1–3 mM range. In addition to free phosphate, phosphate is also present in phosphorylated compounds (DNA, RNA, nucleotides, phosphorylated sugars, lipids, etc.).

Table 5.5. Intracellular Concentrations of Amino Acids

Name	*mM*	*Name (cont.)*	*mM*
Ala	0.2–2.0	Lys	0.1–0.6
Arg	<.05	Met	0.01
Asp	0.4–0.8	Ornithine	0.120
Asn	0.4–0.8	Phe	0.3–0.5
Citrulline	0.036	Proline	0.137
Gln	0.05–4	Ser	0.149
Glu	0.3–12	Thr	0.1–4
His	<0.05–0.09	Tyrosine	0.059
Ile	0.3–0.5	Valine	0.171
Leu	0.1–0.4		

Table 5.6. Approximate Concentrations of Components in a Cellular Environment

	Interstitial fluid (mM)	*Intracellular fluid (mM)*
Na^+	140	6–14
K^+	4.0	100–140
Ca^{2+}	1.2	0.01
Mg^{2+}	0.7	3–20
Cl^-	108	4
HCO_3^-	28.3	10
HPO_4^{2-} $H_2PO_4^-$	2	11
SO_4^{2-}	0.5	1
ATP		1.5
Amino acids	2	8
Carnosine		14
Creatine	0.2	9
Glucose	5	0.05
Lactate	1.2	1.5
Phosphocreatine		40
Protein	0.2	3–4

Many inorganics, including phosphate, potassium, and magnesium, are present at much higher concentrations intracellularly than they are in extracellular fluid or culture medium. As cells grow, they take up nutrients in large enough quantities that they accumulate in the intracellular fluid. It is important to ensure that those inorganic nutrients are also supplied in sufficient quantities. By knowing their intracellular content, the stoichiometric amount required to produce the target biomass can be estimated.

In addition to the major inorganic species (K^+, Na^+, phosphate, Mg^{2+}, and Cl^-), many minute inorganic elements are also constituents of cellular components, including iron, copper, selenium, zinc, and cobalt. Many primarily exist as a prosthetic group of proteins. These elements must also be supplied in enough quantities to generate biomass. The cellular content of those elements is seldom reported and may vary widely among different cell types or even under different culture conditions. For example, the zinc content

is much higher in pancreatic cells than in other cells. Similarly, iron is rich in muscle and red blood cells. Without quantitative data on the cellular contents of those elements, one resorts to titration experiments under defined culture conditions to see whether the supply of those elements is limiting cell growth.

Metabolites

Cells produce and excrete metabolites as they grow. Some metabolites are produced as the product of oxidation of the main nutrient for energy generation (typically glucose), such as lactate, CO_2, and H_2O. Some are produced by metabolic reactions undertaken to balance the amino acids (e.g., alanine, glutamic acid, and asparagine). Others are degradation products of excess amino acids; for example, hydroxybutyrate is produced due to the degradation of isoleucine. The excretion rate of a metabolite is affected by the abundance level of the enzyme of the synthetic pathway, that of the transporter for its excretion, and the concentration of the substrate and the metabolite.

It is virtually impossible to provide cells with every nutrient in a "correct" stoichiometric amount and maintain it at an "optimal intracellular" level so that cell metabolism is at a completely homeostatic state with minimal metabolite excretion except those derived from energy generation (i.e., lactate, CO_2, and H_2O). In other words, the excretion of other metabolites is unavoidable, especially those associated with amino acid conversion and degradation. To close material balance, one has to determine the exact types and quantities of metabolites produced. However, many metabolites are not easily measured as a routine laboratory analysis. Some degradation products are growth inhibitory to cells, so taking a metabolomic approach to identify the potential presence and accumulation of metabolites is certainly worthwhile. By performing material balance on the cell culture system and examining how closely the carbon and nitrogen elements are closed, one can also evaluate the likelihood that additional metabolites may be accumulating that are yet unidentified.

Material Balance on Cell Growth

The process of growing cells and producing a product can be formulated into a stoichiometric equation of "overall biomass synthesis" (Panel 5.3). This equation can be viewed as the composite of all reactions involved in generating energy and synthesizing biomass and product. At the center of the reaction is the biomass. The cell mass is expressed as a chemical formula of elements C, H, N, O, P, S, etc., based upon its elemental composition. Usually, we use a formula of only carbon (C), hydrogen (H), nitrogen (N), and oxygen (O), neglecting the other mineral

Panel 5.3. Example of an "Equation" for Cell Growth

$$C_6H_{12}O_6 + 0.15\ C_{6.14}H_{12.36}N_{1.50}O_{2.08} + 0.34\ C_5H_{10}N_2O_2 + 1.39\ O_2 \rightarrow$$

(glucose) (weighted average of amino acids) (glutamine)

$$2.37\ CH_{1.97}N_{0.26}O_{0.49} + 0.0058\ CH_{1.83}N_{0.14}O_{2.06} + 1.53\ CO_2 + 1.28\ H_2O +$$

(cell mass) (antibody)

$$1.44\ C_3H_6O_3 + 0.16\ NH_3 + 0.13\ C_4H_7NO_2 \qquad \textbf{(Eq. 5-1)}$$

(lactate) (alanine)

elements. This formula presents the mass ratio of those elements. One can arbitrarily assign the stoichiometric numbers to give them different "formula weights." An example of a biomass formula is shown in the cell growth stoichiometric equation (Eq. 5-1, Panel 5.3), in which the stoichiometric number of carbon is chosen to be 1. Others may prefer to assign the formula mass as 100.

The inputs in a cell growth process, i.e., the reactants of the stoichiometric reaction equation of cell growth, include all nutrients consumed by cells and used to proliferate. Since only C, H, N, and O are considered in the stoichiometric equation, we consider only glucose, glutamine, other amino acids, and oxygen. Other minute media components containing C and N, such as vitamins, are neglected. Instead of writing down all amino acids separately, one may use a weighted average derived from the stoichiometric ratio of their consumption.

The outputs, the product side of the stoichiometric equation, include "new" cell mass that is generated as well as the metabolites and product that are excreted. One can write a molecular formula describing the formation of the protein product from its amino acid sequence. The metabolites excreted include lactate, ammonia, some non-essential amino acids, and other excreted minor metabolites.

Growth Stages of Mammalian Cells in Culture

The data of a cell culture process is typically presented as time profiles of the concentrations of cell biomass, nutrients, metabolites, and product. A typical cell growth curve can be divided into a number of growth stages: lag phase, exponential growth phase, stationary phase, and death (or the decline phase) (Figure 5.1). The exponential growth phase is characterized by an exponential increase in biomass, or a period of constant slope in biomass increase on a semi-logarithmic plot over time. For mammalian cells, the period of exponential cell expansion in a culture is often relatively short (typically no more than a 10-fold increase in cell concentration). In some cases, a period of stagnant cell concentration

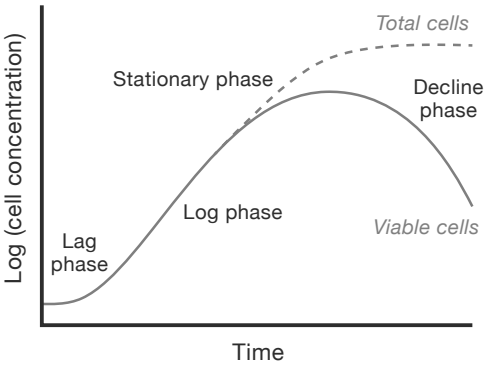


Figure 5.1. A typical growth curve of mammalian cells and growth stages in a suspension culture.

Table 5.7. Doubling Time of Some Culture Cells

Cell type / conditions	*Doubling time*
Mouse embryonic stem cell	~12 h
Human diploid fibroblast (10% FBS)	24–40 h
Human diploid fibroblast (2% FBS)	40–60 h
CHO-K1 (5% FBS or in rich medium)	16–24 h
NS0 cell	16–24 h

may precede the exponential phase, called a lag phase. A lag phase may be caused by using an inoculum from a culture that has already reached a stationary phase, or by using an inoculum grown previously in a vastly different medium. Initiating a culture with a suboptimal cell concentration may also cause poor initial growth. In general, anchorage-dependent cells should be inoculated at a minimum density of 10^4 cells/cm^2 while also maintaining a cell concentration of 10^5 cells/mL. Cells grown in suspension are generally started at about 10^5 cells/mL, but are at a higher level for very small blood cells such as natural killer cells.

The exponential phase is marked by a constant cell growth rate, or doubling time (the time duration that cell biomass or cell number doubles). The growth rate of a cell line is dependent on the medium, temperature, pH, and many other physical and chemical conditions. Additionally, most cell lines, cell strains, or tissue cells require a set of growth factors or cytokines for growth. The growth rate is affected by the levels of the required growth factors. The maximum growth possible under most favorable conditions for different cell lines and cell types spans over a wide range, from a fast rate of 12 h for mouse embryonic stem cells to ~24 h for human diploid fibroblasts (Table 5.7). Cells commonly used in therapeutic protein production double every 16–30 h.

After the rapid growth period, due to nutrient depletion or the accumulation of metabolites, the growth rate diminishes and the cell concentration stops increasing. This is referred to as the stationary phase. A stationary phase may also reflect a balance between cell growth and death. In the latter case, the growth curve is characterized by a constant viable cell concentration, along with an increasing concentration of dead cells.

Once cell viability begins to decrease, the culture is considered to be in a decline phase. The growth behavior at a late stage of culture often varies, depending on whether cells are adherent or in suspension. Many anchorage-dependent cells grow substantially slower once cell density on the surface approaches confluence. They can also sustain a confluent cell density over a period of days without entering the death phase.

Conversely, cells grown in suspension, especially those with an inherently strong receptor-mediated apoptotic mechanism, often enter the death phase soon after the viable cell concentration peaks.

Kinetic Description of Cell Growth & Product Formation

Specific Rates

A CELL CULTURE PROCESS can be characterized by how the concentrations of cell biomass, nutrients, and product change over time. From these data of time profiles of concentrations, one can determine the rates of change of those concentrations, how those rates relate to each other, and how active or productive the process is.

The rate of concentration change of a culture component is determined from the slope of its concentration profile over time and is referred to as the volumetric rate. Eq. 5-2, Panel 5.4 shows the volumetric rate for cell growth. One can similarly express the volumetric rates of nutrient consumption or product formation, although they are not frequently used. A culture that is quickly accumulating a large number of cells per culture volume (i.e., has a high volumetric growth rate) may have started from a high concentration of cells or from cells that are more actively growing. Hence a more useful quantity is the specific rate, which describes how active cells are.

Usually, cell volume constitutes only a very small fraction of the volume in a suspension cell culture. The volumetric rate is essentially based on the liquid volume. In some cases, such as with high-density solid microcarrier culture, the volume occupied by solid beads is large; thus, liquid volume and culture volume are not equal. Usually the concentrations of nutrients, metabolites, and product are measured based on liquid volume, not on culture or reactor volume. In such cases, the "volume" used for different kinetic parameters must be clearly stated.

Another set of descriptors, called the specific rate, is normalized to a per cell basis. These activity parameters describe how active each unit of the cell is for activities like making new biomass (specific growth rate), consuming glucose (specific glucose consumption rate), or producing lactate (specific lactate production rate).

The speed at which cells generate new biomass is linearly dependent on the number of cells in culture, as shown in Eq. 5-3 (Panel 5.4). The proportionality (or the rate constant) is called the specific growth rate, μ. This quantitative description allows one to compare the speed of growth among different cells or cells under different culture conditions. It has a

Panel 5.4. Specific Rates Describing Growth Kinetics

Growth Rate (G): Change in cell concentration per unit time (cell number/L-h; or g cell/L-h)

$$G = \frac{dx}{dt} \qquad \textbf{(Eq. 5-2)}$$

Specific Growth Rate (μ): Cell number/cell-h or g cell/g cell-h

$$\frac{dx}{dt} = \mu x \qquad \textbf{(Eq. 5-3)}$$

$$\mu dt = \frac{1}{x} dx \qquad \textbf{(Eq. 5-4)}$$

Doubling Time

$$\ln x = \mu t \qquad \textbf{(Eq. 5-5)}$$

$$x = x_0 e^{\mu t} \qquad \textbf{(Eq. 5-6)}$$

$$\ln \frac{x_2}{x_1} = \mu \left(t_2 - t_1 \right) \qquad \textbf{(Eq. 5-7)}$$

$$t_d = \frac{\ln 2}{\mu} = \frac{.693}{\mu} \qquad \textbf{(Eq. 5-8)}$$

Specific Nutrient Consumption Rate: g nutrient/g cell-h

$$q_s = \frac{-1}{x} \frac{ds}{dt} \qquad \textbf{(Eq. 5-9)}$$

Specific Product Formation Rate

$$q_p = \frac{1}{x} \frac{dp}{dt} \qquad \textbf{(Eq. 5-10)}$$

unit of the inverse of time. For mammalian cells and microorganisms in culture, it is normally expressed in h^{-1}. This is in contrast to the volumetric cell growth rate, which has units of cells-$L^{-1}h^{-1}$. Note that the quantity of cells may be described in terms of cell mass, cell number, or some cellular component, depending on the method used to measure cell concentration. In the specific growth rate, the cell quantity descriptor is not present. However, a specific growth rate determined using different cell measurements may give somewhat different values, because cell size, cell mass, and cellular content may not be related to one another in the same proportion under different culture conditions. For example, under some conditions cells may not divide and increase in number, but expand in cell volume and biomass. So the specific growth rate based on the cell number measurement is different from that obtained with cell mass.

The cell doubling time is used frequently to describe how quickly cells grow. By separating the variables x and t in Eq. 5-3 into two sides of the equation (Eq. 5-4) and integrating with respect to time and cell concentration, respectively, one obtains the relationship between cell concentration and time, given a constant specific growth rate (Eqs. 5-5 and 5-6). The doubling time is the time it takes to develop a 2-fold increase in cell concentration (Eq. 5-8).

A descriptor for the activity of nutrient (often also called substrate) consumption, or the specific rate of nutrient consumption, can be similarly defined as shown in Eq. 5-9. Note that a negative sign is given since the nutrient concentration decreases over time. The specific nutrient consumption rate is obtained by dividing the volumetric nutrient

consumption rate (ds/dt) by the cell concentration. Similarly, a specific product (or metabolite) formation rate can be defined (Eq. 5-9).

In some cases, non-viable cells constitute a significant portion of the cell population. A death term is added to the cell concentration balance (Eq. 5-11, Panel 5.5). Note that x represents viable cells, and x_d denotes dead cells. The total cell concentration is the sum of x and x_d (Eq. 5-12). The dead cells arise from viable cells at a frequency described by the specific death rate, μ_d. Using the definition shown in Panel 5.5, the specific growth rate is calculated from the rate of change of the total cell concentration. If one uses the viable cell concentration to calculate the growth rate (dx/dt), the resulting specific rate (Eq. 5-12) is $\mu - \mu_d$.

Cell lysis, in which dead cells further lyse and become invisible in cell counting, may occur in culture. Their numbers are sometimes estimated from the measurement of the release of cellular content such as DNA or an intracellular enzyme. Cell lysis is assumed to occur from dead cells (Eq. 5-14). To describe the growth kinetics of such a culture, one can also include a cell lysis term (Eq. 5-15).

Panel 5.5. Specific Rates in the Presence of Cell Death

Cell Growth in Culture

$$\frac{dx_d}{dt} = \mu_d x \qquad \textbf{(Eq. 5-11)}$$

$$\frac{dx}{dt} = \mu x - \mu_d x \qquad \textbf{(Eq. 5-12)}$$

$$x_t = x + x_d \qquad \textbf{(Eq. 5-13)}$$

Specific Rate

$$\mu = \frac{1}{x}\frac{dx_t}{dt} \qquad \textbf{(Eq. 5-14)}$$

$$q_s = \frac{-1}{x}\frac{ds}{dt} \qquad \textbf{(Eq. 5-15)}$$

With Cell Lysis

$$\frac{dx_d}{dt} = \mu_d x - \lambda x_d \qquad \textbf{(Eq. 5-16)}$$

$$\frac{dx}{dt} = \mu x - \mu_d x - \lambda x_d \qquad \textbf{(Eq. 5-17)}$$

Panel 5.6. Yield Coefficient and Stoichiometric Ratios

Yield of Biomass on Substrate

$$Y_{x/s} = \frac{dx}{ds} \approx \frac{\Delta x}{\Delta s} \qquad \textbf{(Eq. 5-18)}$$

Yield of Product on Substrate

$$Y_{p/s} = \frac{dp}{ds} \approx \frac{\Delta p}{\Delta s} \qquad \textbf{(Eq. 5-19)}$$

Stoichiometric Ratio of Two Substrates

$$\alpha_{s_1/s_2} = \frac{\Delta s_1}{\Delta s_2} \qquad \textbf{(Eq. 5-20)}$$

Stoichiometric Ratio of Lactate to Glucose

$$\alpha_{L/G} = \frac{\Delta L}{\Delta G} \qquad \textbf{(Eq. 5-21)}$$

The calculation of the other specific rate must also account for cell death (Eq. 5-16). The cell concentration used to calculate the specific nutrient consumption or product formation rate is the viable cell concentration (Eq. 5-17). In other words, one assumes that dead cells are metabolically inactive.

Stoichiometric Ratio and Yield Coefficient

In evaluating the performance of a process, we assess the efficiency of material conversion, i.e., how much of a raw material is converted into the product. The performance is then compared to a target value for further optimization.

A yield coefficient is frequently used to describe the efficiency of raw material conversion to cell mass or product in microbial fermentation, where the cost of raw materials is often a very significant fraction of the production cost. The yield coefficient is given a symbol $Y_{x/s}$ or $Y_{p/s}$, for cell mass or product, respectively (Eqs. 5-18 and 5-19 in Panel 5.6), and is based on the consumption of a particular substrate. It is the ratio of the quantity of the product or cell produced to that of the substrate consumed. A yield coefficient for a cell can be based on different input materials, e.g., glucose or other nutrients.

Yield coefficients are rarely used in animal cell culture processes. Cell concentration in a typical animal cell culture process is seldom measured in mass. Furthermore, the medium components that are used in large quantities by cells (glucose and amino acids) are not the top contributors to the cost of goods.

The stoichiometric ratio of various nutrients and metabolic products is more frequently used in cell culture processes. They are the ratio of the amount of different nutrients consumed or metabolites produced over a time period in culture (Eqs. 5-20 and 5-21). Under different metabolic conditions or in different growth stages, cells utilize various nutrients differently and change the number of different metabolites produced. Changes in the stoichiometric ratios of various nutrients and metabolites are indicators of alterations in metabolism (Panel 5.7). Furthermore, knowing the stoichiometric ratio of nutrient consumption is essential in devising a sound nutrient feeding scheme in fed-batch and perfusion cultures.

Panel 5.7. Description of the "State" of the Cultures

- Physiological state
  - There is no unique or universal definition for the physiological state
  - In general, the kinetic parameters described above are sufficient to describe it
- Metabolic state
  - Can be described by the stoichiometric ratios

For example, the stoichiometric ratio of lactate to glucose (e.g., the ratio of the amount of lactate produced to that of glucose consumed) is a strong indication of whether the energy metabolism is at a highly glycolytic or highly oxidative state. If most

glucose is channeled through glycolysis to lactate, the ratio is close to two moles of lactate per mole of glucose. Conversely, if most glucose is directed toward the TCA cycle for aerobic oxidation, the ratio will be close to zero, while the stoichiometric ratio of oxygen to glucose will be closer to six moles of oxygen per mole of glucose.

The stoichiometric ratio and yield coefficient, based on a given pair of compounds, can be expressed in different units, e.g., mol/mol or g/g.

Integral Cell Concentration

In the early days of therapeutic protein development, the quantitative assay of product was often performed by specialized scientists. The measurement usually took a substantial amount of time that delayed process development. To facilitate the assessment of various process conditions for production, integral cell concentration was used. The product accumulation rate in a culture can be described by multiplying the specific product formation rate by cell concentration (Eq. 5-22 in Panel 5.8). Integration over the culture period from t_0 to t_f obtains the product concentration p_f at t_f (the starting product concentration is 0) (Eqs. 5-23 and 5-24). If q_p is constant, one can take it out of the integral. One can see that the final product concentration is proportional to the integral of cell concentration (Eq. 5-25). In a plot of cell concentration (x) vs. time, the integral cell concentration is the area under the curve of the cell concentration curve (Figure 5.2). Hence, integral cell concentration was used as an indication of the product accumulated in culture. This is still in practice widely. However, one should be aware that q_p is often not constant. It is a better practice to examine q_p at different stages of the culture for process optimization.

Panel 5.8. Integral Viable Cell Concentration

$$\frac{dp}{dt} = q_p x \qquad \textbf{(Eq. 5-22)}$$

$$dp = q_p x\, dt \qquad \textbf{(Eq. 5-23)}$$

$$\int_0^{p_f} dp = \int_{t_0}^{t_f} q_p x\, dt \qquad \textbf{(Eq. 5-24)}$$

$$p_f = q_p \int_0^{t_f} x\, dt \qquad \textbf{(Eq. 5-25)}$$

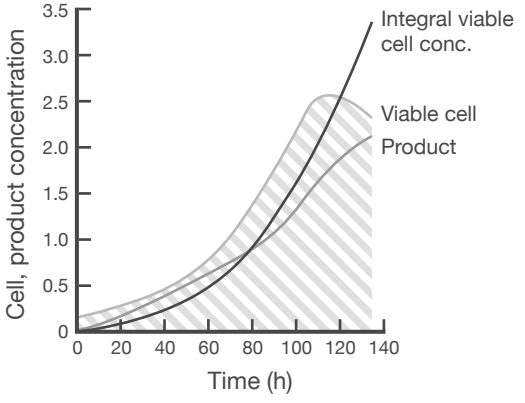


Figure 5.2. Cell and IgG product concentration in a batch culture and the viable integral cell concentration over time.

Kinetic Model of Cell Growth

In this section we will discuss using mathematical models to conduct computer experiments to evaluate process strategies. The topic would have seemed unfeasible only a few years ago. There are different levels of using a mathematical model for cell culture process development.

First, a model can be used to summarize a large volume of experimental data. In describing a process, we plot the concentrations of cells, key substrates/metabolites, and product (the core components of cell culture processes) over time. The data or their plots are extremely difficult to describe in words. By converting the data or plots into a set of equations or a mathematical model, regardless of whether the model is empirical or mechanistic, the behavior of the data can then be recreated by anyone who is given the model and value of the parameters.

A model can also be used to explore ideas and test hypotheses. When we study a physical system and its behavior, we first develop a verbal description of the system. We may then propose a hypothesis about the system's behavior, also verbally. The verbal description of the system and hypothesis can be translated into a mathematical form. The mathematical model can then be used to explore the system's possible behavior under different conditions. One may be able to compare the model simulation and experimental results and use the insight to refine the hypothesis. Ultimately, if one has a well-constructed model, one can use the model to predict the behavior of the systems. If the model is developed based on a mechanistic understanding of the system, it may be used to predict regions of parameter space that have not been experimentally tested previously. It can also be used to optimize or control the dynamics of the system.

Monod Model

At the core of any model for a cell culture system is the description of how the cell growth rate changes with a changing environment. A number of mathematical models have been used to describe mammalian cell growth in culture. Most are based on Monod-type models that were traditionally used to describe microbial growth. Verbally, the Monod model states that the growth rate of microorganisms increases with increasing limiting nutrient concentration, but eventually reaches saturation (maximum growth) as the nutrient level keeps increasing. The model was first introduced by Jacques Monod to describe the growth of *E. coli* cells on glucose. Monod then expressed the statement in a mathematical form.

The Monod model uses two parameters, μ_{max} and K_s, to describe the relationship between the specific growth rate, μ, and the limiting substrate concentration, s (Eq. 5-26, Panel 5.9, Figure 5.3). The equation gives a saturation type of behavior; μ increases with the limiting substrate

Panel 5.9. Monod Model

$$\mu = \frac{\mu_{\max} s}{K_s + s} \qquad \textbf{(Eq. 5-26)}$$

when $s >> K_s$, $\mu \rightarrow \mu_{\max}$

when $s << K_s$, $\mu \rightarrow \mu_{\max} s/K_s$

- An empirical model for first-order approximation of cell growth
- Under most process conditions, growth is not limited by nutrient availability

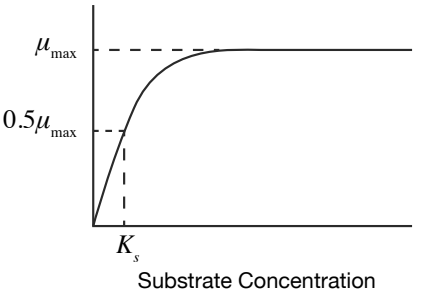


Figure 5.3. The specific growth rate as a function of growth-rate-limiting substrate concentration as described by the Monod growth model.

concentration until it reaches a maximal value, $\mu_{\max}$, asymptotically. At low concentrations, increasing the rate-limiting nutrient concentration increases the specific growth rate linearly. At very high concentrations, the specific growth rate is constant at μ_{max}.

The Monod model is an empirical model, where one uses experimental data to fit the model and determine the value of $\mu_{\max}$ and K_s. Nevertheless, it depicts the growth curve of microbes well. When glucose is imposed as the limiting nutrient for mammalian cells in culture, it adequately describes the growth rate dependent on glucose concentration. However, under most culture conditions as well as *in vivo*, glucose is not the limiting nutrient. Instead, the growth rate is often dependent on the concentration of growth factors and subject to the regulation of signaling pathways. In industrial cell culture, the growth is more likely to be influenced by the accumulation of metabolites. Nevertheless, the Monod model is simple and has its merits for exploring different culture conditions.

Models for Multiple Nutrients and Adherent Cells

The Monod model describes growth dependence on a single limiting nutrient. Mammalian cells require multiple nutrients for proliferation. In addition to glucose, cell growth also requires glutamine, other amino acids, and many other nutrients. Although most are provided in excess and are not limiting, under some conditions the growth or metabolic behavior is affected by more than one nutrient simultaneously. Monod models are modified to describe the dependence of growth rate on multiple nutrients. Most common modifications incorporate multiplicative terms involving multiple substrates. Some also include the inhibitory effect of metabolites on the growth rate.

Panel 5.10. Multiplicative Saturation Kinetics Model

$$\mu = \mu_{\max} \cdot \frac{s_1}{K_1 + s_1} \cdot \frac{s_2}{K_2 + s_2} \qquad \textbf{(Eq. 5-27)}$$

$$\mu = \mu_{\max} \cdot \frac{s_1}{K_1 + s_1} \cdot \frac{s_2}{K_2 + s_2} \cdot \frac{K_{iA}}{K_{iA} + A} \qquad \textbf{(Eq. 5-28)}$$

In the multiplicative model for cell growth, two substrates, s_1 and s_2, are considered to influence the growth rate (Eq. 5-27, Panel 5.10). Each substrate has its corresponding half-saturation constant K_s. In most common applications, the two substrates are glucose and glutamine. The model can be extended to consider metabolite inhibition (Eq. 5-28). In the example shown in Eq. 5-28, A is an inhibitory metabolite, like lactate.

Panel 5.11. Growth Rate Dependence on Cell Density of Adherent Cells

$$\mu = \mu_{\max}\left(1 - \frac{y}{y_{\max}}\right) \qquad \textbf{(Eq. 5-29)}$$

Many cell types, including some used in viral vaccine manufacturing, are adherent. Their growth rate is not only affected by nutrient and metabolite concentrations, but also by the cell density on the surface to which they adhere. At low cell densities, cells grow at a maximum rate; as the cell density approaches "confluence" (i.e., the maximum density), the growth rate decreases (Eq. 5-29, Panel 5.11).

Modeling Productivity

The kinetics of product formation in microbial systems are typically described using an empirical formula of proportionality to the specific growth rate (Eq. 5-30, Panel 5.12). The specific productivity, q_p, is considered to be influenced by two factors: a "growth-associated" term, α, which describes dependence on the specific growth rate; and a "non-growth associated" term, β. Depending on the relative magnitude of α and β, the production can be positive-, negative-, or non-growth associated.

Panel 5.12. Specific Product Formation Rate: Dependent on Growth Rate

$$q_p = \alpha\mu + \beta \qquad \textbf{(Eq. 5-30)}$$

Such classifications of specific productivity have limited applicability to mammalian cell culture systems. In general, the specific productivity of cell culture processes is relatively insensitive to the growth rate. For some recombinant proteins, the productivity is somewhat higher in the stationary phase of fed-batch cultures. At that slow-growing stage, many factors, including osmolality, lactate, and CO_2 concentration, have all deviated from optimal growth conditions. However, it is generally thought that the dominating factors that increase the productivity in the late stage of a culture are related to the stress conditions but not the growth rate. With a sufficient amount of data, one may use an

empirical model to describe the specific productivity as a function of the stress index that considers different factors contributing to stress, such as osmolality and the concentration of some metabolites, like butyrate, hydroxybutyrate, and lactate.

Modeling a Cell Culture System

An important objective of developing a mathematical model for a cell culture system is being able to use it to predict culture behavior by testing different process conditions *in silico*, especially for conditions that cannot be easily carried out experimentally. In the section below, we will discuss the basic considerations in establishing a model for a cell culture system.

Elements of a System Model

The first step to building a model is identifying the pivotal variables of the system (Panel 5.13). In general, they include the concentrations of cells, glucose, lactate, and other factors affecting the specific growth rate and specific productivity. One then wants to develop a description of how those variables change over culture time due to their dynamic interactions with one another. Because changes in both those variables and their interactions are dynamic, one uses a set of differential equations to describe their dynamics. The second step is thus to establish the model or the system of equations of the cell culture system. Next, one specifies the interactions of the variables using mathematical descriptions and uses the experimental data to quantify the parameters used in the model so one can solve the equations and simulate the system behavior under different conditions.

Panel 5.13. Necessary Components for a Mathematical Description of a Cell Culture Process

- A description for:
  - Cell growth (and death)
  - Product formation
  - Nutrient utilization
- Growth model
  - The dependence of the specific consumption rate on the "controlling variable" (growth rate, nutrient concentration, etc.)
- Product formation
  - The dependence of the specific production rate on the "controlling variable"
- Experimental data
  - The model will have some parameters (e.g., half-saturation constants). The experimental data are used to determine the value of those parameters.
- Material balance equations
  - For "state variables" (the concentrations of cells, nutrients, product, inhibitors, etc.)

Balance equations on pivotal variables

After the pivotal variables for the system being considered are identified, a material balance equation for each variable is written. Shown in Panel 5.14 are example equations for a batch system. The rate of change of cell concentration (left-hand

Panel 5.14. A Cell Growth Model Incorporating Interactions, Growth, and Metabolism

Cell Balance

$$\frac{dx}{dt} = \mu x = \frac{\mu_{\max}}{(K_L + L)} \qquad \textbf{(Eq. 5-31)}$$

Glucose Balance

$$\frac{dG}{dt} = -q_G x = -q_G(\mu, L)x \qquad \textbf{(Eq. 5-32)}$$

Product Balance

$$\frac{dp}{dt} = q_p x \qquad \textbf{(Eq. 5-33)}$$

Lactate Balance

$$\frac{dL}{dt} = q_L x = q_L(\mu, G, L)x \qquad \textbf{(Eq. 5-34)}$$

side of Eq. 5-31) is the consequence of only cell growth (i.e., the specific growth rate multiplied by cell concentration) since there is no input or output for a batch culture. For a fed-batch or continuous culture, the balance equations will include additional terms to account for inputs and outputs. This will be discussed in Chapters 9 and 10. Equations are shown for the balance of glucose, lactate, and a product (Eqs. 5-32, 5-33, and 5-34). Again, because neither input nor output are present in a batch system, the balance is only attributed to the consumption of glucose and the production of lactate.

The reason that one includes all those variables is their interactions. In other words, the concentration of one variable affects the rates of change of other variables. For example, the specific growth rate may be affected by the concentrations of glucose and lactate. The cell concentration affects the rates of change of both glucose and lactate; hence the three variables (concentrations of cells, glucose, and lactate) are mutually interacting. If the specific production rate is affected by glucose and lactate, but the product production does not affect the specific rates of growth, glucose consumption, and lactate production, then the balance equation for the product does not need to be included in the system. One can first solve the time profiles of cells, glucose, and lactate, and then compute the product profile. On the other hand, if the product is a cytokine that has growth inhibitory effects, then the balance equation needs to be included in the system of differential equations.

Kinetic model for growth and metabolism

A model that describes the growth kinetics of cells in a culture therefore consists of material balance equations of the pivotal variables, including cell mass, nutrients, and metabolites (glucose and lactate equations). Embedded in the system of equations are quantitative descriptions of the relationship between the specific rate of growth, nutrient consumption, metabolite production, and nutrient and metabolite concentrations, or other factors affecting those rates. Once those relationships and the appropriate initial conditions (i.e., the initial concentrations of cell mass, glucose, lactate, and product) are specified, the system of equations can be solved and the behavior of the culture simulated.

The key to developing a model of a cell culture system is thus the description of the relationships between growth and metabolism. Approaches largely fall into two types: one employs empirical relationships determined by fitting experimental data to the model, and the other uses a mechanism-based metabolism model but relies on empirical modeling to describe the relationship between the growth rate and the environmental variables.

Empirical Models

Our current understanding of growth rate control does not permit us to develop a mechanistic model for cell growth that is responsive to environmental chemical and physical conditions. A Monod model or a modified Monod model is commonly used to tie the balance equations of cell and substrate together. The model typically describes substrate consumption as being related to growth rate empirically, for example, by using a proportional relationship. Such a model, when used within the range of experimental observation, can describe cell growth and the substrate profile. One may extend the model to describe the effect of inhibitory metabolites and osmolality on the growth rate. An empirical model is useful in simulating culture performance under conditions that do not deviate hugely from those previously observed, but its predictive value is limited.

Hybrid System of Mechanistic/Empirical Models

In the past two decades, scientists have developed extensive understanding of the kinetic behaviors of nearly all the enzymes involved in energy metabolism. Additionally, our knowledge of the regulation of those enzymes at transcription, translation, and activity levels is rapidly expanding. It is now possible to develop a kinetic model that describes a cell's energy metabolism. Material balance equations for reactions intermediate of energy metabolism, complete with enzymes' kinetic expressions, can be established. The uncertainty in estimating the level of each enzyme involved can be dealt with by using the transcriptome data as a first-order estimation followed by a parameter estimation using experimental data. A mechanistic model was used to describe metabolic shift from a high glycolytic flux state to a low flux state, a phenomenon frequently seen in fed-batch culture and which is tied to a high productivity in recombinant protein production.[1] Such an intracellular metabolic model can be linked to an extracellular model that describes the concentrations of cells, glucose, and lactate as described above. The linkage is mediated by the transporters for glucose, lactate, etc. Such an integrated intracellular and reactor model invariably deals with an intracellular model of metabolism in a fast time scale and a reactor model that is on a much slower time scale (Figure 5.4). The integrated model, hybrid model

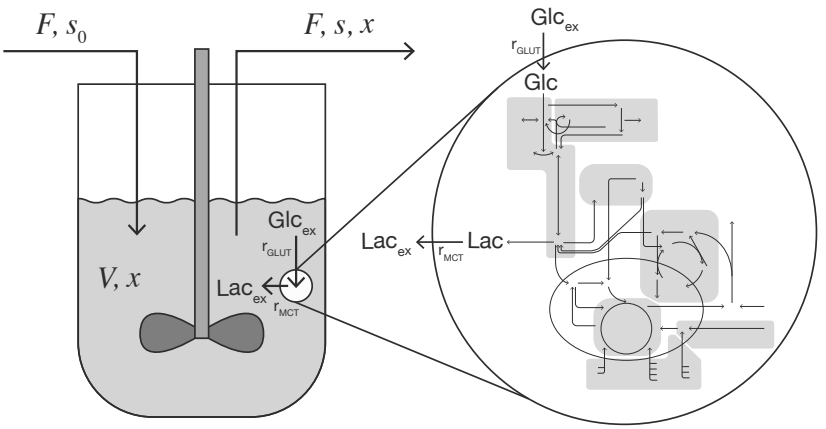


Figure 5.4. Depiction of a hybrid multiscale model for cell growth in culture.

of empirical growth, and mechanistic metabolic model will be multiscale, dealing with both the fast time scales of the metabolic reactions and the slow time scales related to the growth and change of chemical environments in the reactor. A multiscale hybrid model has been applied to explore the multiple steady state behavior in continuous culture.[2]

Concluding Remarks

Stoichiometric and kinetic relationships among process variables are important in describing the behavior of cell culture processes. They provide a framework for quantitative comparison of different processes. Empirical growth kinetic models allow for simulation of a culture's kinetic behavior under different growth conditions. Advances in metabolic science have enabled the development of mechanistic metabolic models. A hybrid multiscale model integrating empirical growth kinetic descriptions and a mechanistic metabolic computation framework will enhance our ability to optimize the cell culture process through *in silico* experiments. It is a goal that is attainable and will gain more momentum in the coming years.

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2. Yongky, A., et al., *Mechanism for multiplicity of steady states with distinct cell concentration in continuous culture of mammalian cells.* Biotechnology & Bioengineering, 2015. **112**(7): p. 1437–45.