Introduction

In the past decade, there has been an increasing interest in the continuous manufacturing of pharmaceuticals. The initial focus, which was on chemical drugs, has extended to protein biologics in recent years. The drive was prompted by the success of continuous processes in the chemical industry. A large proportion of chemical reactors are operated in a continuous fashion: a feed stream continuously brings in the reactants while an effluent stream continuously draws the reactor contents out. A continuous reactor offers a number of attractive features. It uses the reactor more efficiently than a batch process by minimizing equipment turnover time and process start-up time. It provides a continuous product stream from the reactor at a product concentration comparable to a batch process, and it can be operated at a steady state over a long time to achieve a higher throughput than a batch process.

Many enzymatic biotransformation, waste treatment, and biodegradation processes have long been operated continuously. However, continuous culture is still not the dominant process type in microbial and cell culture bioprocessing.

The vast majority of biochemical processes that involve microbial or animal cell cultivation are batch processes for many reasons. Unlike the catalysts used in the chemical industry whose activities are stable over time, cells may mutate or change epigenetically. As a result, the makeup of the population, its productivity, and even the product quality changes over time. Additionally, the risk of microbial contamination makes long operations susceptible to failure. It is also notable that the current product capture and purification operations are all designed for batch

mode. Even if the production bioreactor is operated in a continuous mode, the overall process is still cyclical and does not realize all of the advantages of a continuous process as seen in the chemical and other manufacturing industries.

A continuous culture is constrained by the maximum flow rate at which it can operate, as the continuous fluid flow must not wash out cells faster than cell growth can replenish them. On the other hand, cells in culture produce growth inhibitors and metabolites that must be continuously removed by media replenishment in order to avoid growth inhibition. For cell culture processes, at a flow rate that meets both constraints, the achievable cell and product concentrations are too low to be economically viable. To overcome this shortcoming, a cell recycling system is added to the continuous culture. By recovering cells from the effluent flow and returning them to the reactor, one can operate a continuous culture beyond the natural limitation of its dilution rate (the flow rate divided by the bioreactor volume). With a higher cell concentration in the reactor, the overall throughput of the reactor is also higher.

When cell culture was first adopted as the production vehicle for biopharmaceuticals, continuous operation was explored as an industrial process. Researchers realized that the growth of mammalian cells in batch culture was impeded by lactate and ammonium accumulation. Thus, a continuous process alleviates growth inhibition by removing metabolites through a continuous flow of medium. However, the high cost of continuously removing expensive medium with only a low concentration of product made continuous culture economically unaffordable.

To enable continuous operation, cells are retained in the reactor while the medium flow flushes out metabolites. This can be accomplished by either immobilizing cells on solid particles, which prevents them from being flushed out by the medium flow; or by separating cells from the effluent stream and recycling them back to the reactor. Thus, the general idea is continuous culture with cell retention. These processes are often called perfusion, which is reminiscent of the procedure of flowing fluid through an organ or tissue.

Table 10.1. Marketed Cell Culture Products Produced by Perfusion Processes

Product	*Company*
RecombinantTM, Antihemophilic factor (recombinant), (factor VIII)	Baxter
Kogenate-FS (factor VIII)	Bayer
Aldurazyme Naglazyme	BioMarin
ReoPro (IgG Fab Fragment) Remicade (IgG1) Simponi (IgG1)	Centocor (Janssen)
Xigris (Protein C)	Eli Lilly
Cerezyme Fabrazyme Myozyme/Lumizyme	Genzyme (Sanofi)
Gonal-F (follicle-stimulating hormone)	Serono (EMD)
Vpriv (velaglucerase alfa) Replagal (agalsidase alfa)	Shire (Takeda)
ReFacto (factor VIII)	Wyeth (Pfizer)

Panel 10.1. Continuous Cell Culture with Cell Retention

Use of Perfusion Culture

- For products that are labile or produced at very low levels
- Requires in-house expertise on handling continuous operation
- Recent advances in cell retention technology and the use of media with less complex components enabled its wider adoption

Potential Advantages of Perfusion Culture

- Possible steady-state operation
- More consistent product quality
- Lower metabolic levels and higher cell viability than at the conclusion of fed-batch culture

A number of biotherapeutic proteins are produced by perfusion processes (Table 10.1). Some of these products can be produced with a fed-batch process. The selection of the process mode is also dependent on the expertise available in-house and many other factors (Panel 10.1). If the product is labile and degraded or otherwise inactivated over time, a perfusion culture is certainly the process of choice. For products that accumulate only at very low concentrations, a perfusion process may also present a competitive advantage over a batch or fed-batch process by reducing the equipment turnover time. The advances in cell separation technology have greatly expanded the space of continuous culture in cell bioprocessing.[1] These advances have made a long-term perfusion culture achievable for many products. This, combined with the commercial availability of medium-production-scale disposable stirred tank bioreactors, has led to perfusion culture and hybrid perfused fed-batch culture becoming more widely adopted.

Analysis of Continuous Culture and Steady State

Simple Continuous Culture

We will use an idealized, continuous, well-mixed bioreactor to explain the basic concept of continuous culture and steady state, even though a simple continuous culture is rarely practiced. The stirred tank bioreactor receives a continuous feed stream and releases a continuous effluent stream consisting of medium and cells from the bioreactor (Figure 10.1a). Because of its "well-mixed" nature, the substrate from the feed stream is instantaneously mixed with the content of the bioreactor, and the effluent stream has the same composition as the content of the bioreactor. The flow rates of the feed and effluent streams are the same (i.e., $F_{in} = F_{out} = F$) to keep the reactor volume constant.

The material balance equations for cells and the rate-limiting substrate (Eqs. 10-1 and 10-5, Panel 10.2) reflect the balance of the amount of cell mass or substrate carried into and out of the reactor by the fluid flow. They also account for changes to cell or substrate concentrations caused by cell growth and nutrient consumption (ignoring cell death).

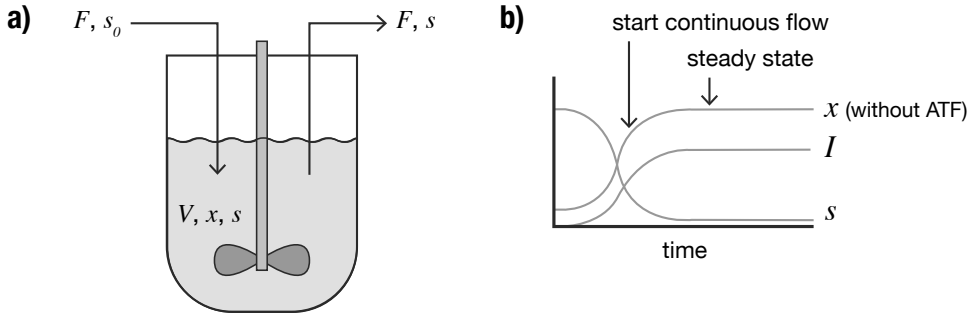

Figure 10.1. (a) A continuous culture bioreactor. (b) The starting up of a continuous culture to reach a steady state.

The flow rate into the reactor divided by the reactor volume is called the dilution rate (D, Eq. 10-2). Because the volume of the culture is constant, the material balance equation can be based on the concentration rather than on the total mass quantity in the culture (Eq. 10-3). The increase in cell mass due to growth is expressed as the multiplicative product of the specific growth rate (μ) and cell concentration (x). The consumption rate of the substrate is the cell concentration multiplied by the specific substrate consumption rate (q_s, Eq. 10-6).

Typically, a culture is started in batch mode. When the cell concentration approaches its maximum, continuous flow of the feed and effluent streams begins (Figure 10.1b). After some time, the cell concentration and substrate concentration become invariant. The culture is then regarded as having reached a steady state, as the most important culture variables (state variables) are no longer changing ($dx/dt = ds/dt = 0$). At a steady state, the specific growth rate and the dilution rate are equal (Eq. 10-4). This is a very important property of continuous culture. If a

Panel 10.2. Material Balance for a Continuous Culture

$$\frac{dxV}{dt} = V\frac{dx}{dt} = Fx_0 - Fx + \mu xV \qquad \textbf{(Eq. 10-1)}$$

$$D = \frac{F}{V}\text{, cancel } V \qquad \textbf{(Eq. 10-2)}$$

$$\frac{dx}{dt} = \mu x - Dx \qquad \textbf{(Eq. 10-3)}$$

At steady state, $0 = \mu x - Dx$,

$$\therefore \mu = D \qquad \textbf{(Eq. 10-4)}$$

$$\frac{dsV}{dt} = Fs_0 - Fs - q_s xV \qquad \textbf{(Eq. 10-5)}$$

Divide by V and apply Eq. 10-2.

$$\frac{ds}{dt} = D\left(s_0 - s\right) - q_s x \qquad \textbf{(Eq. 10-6)}$$

At steady state,

$$D\left(s_0 - s\right) = q_s x \qquad \textbf{(Eq. 10-7)}$$

$$x = \frac{D\left(s_0 - s\right)}{q_s} \qquad \textbf{(Eq. 10-8)}$$

$$\frac{dI}{dt} = \alpha_{I/s} q_s x - DI \qquad \textbf{(Eq. 10-9)}$$

$$\text{At steady state, } I = \frac{\alpha_{I/s} q_s x}{D} \qquad \textbf{(Eq. 10-10)}$$

culture system is to be operated at a steady state, with cells comprising part of the effluent stream, then the cells must be growing and the specific growth rate must be equal to the dilution rate. Because of its simplicity, continuous culture has been an excellent tool for studying the kinetics of cell growth and metabolism.[2]

The cell concentration that can be achieved using a given feed substrate concentration at steady state can be calculated (Eqs. 10-7 and 10-8). In cell culture processes, inhibitor accumulation affects cell growth. Eq. 10-9 considers the case that the production of inhibitors is related to the consumption of substrate ($\alpha_{I/s}$), as evidenced in glucose consumption leading to lactate production. The concentration of the inhibitor (I) is determined by the cell concentration and the dilution rate (Eq. 10-10).

A continuous culture at steady state

To illustrate the steady state concept, we will use a simple Monod model to describe the relationship between the growth rate and the substrate concentration (Figure 10.2a, Eq. 10-11, Panel 10.3). We further assume that the specific substrate consumption rate is proportional to the growth rate (Eq. 10-12). $Y_{x/s}$ in Eq. 10-12 is the yield coefficient, i.e., the number of cells produced for each unit amount of substrate consumed. According to the Monod model, under a given substrate concentration (s) there is only one specific growth rate (μ). The material balance on cells and substrate gives a system of two equations that describes all steady states (Eqs. 10-13 and 10-14). With a given cell line, μ_m, $Y_{x/s}$, and

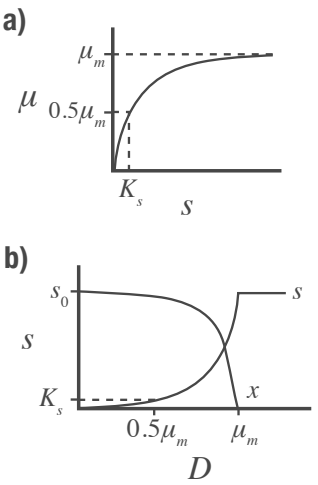

Figure 10.2. (a) The Monod model of growth kinetics. (b) The steady state cell and substrate concentration profiles of a continuous culture with Monod growth kinetics.

Panel 10.3. Steady State of a Continuous Culture with Monod Growth Kinetics

Assume $\mu = \dfrac{\mu_m s}{K_s + s}$ (Monod model) **(Eq. 10-11)**

and $q_s = \dfrac{\mu}{Y_{x/s}}$ **(Eq. 10-12)**

From Eq. 10-4:

$$\frac{\mu_m s}{K_s + s} = D \Rightarrow s = \frac{DK_s}{\mu_m - D} \quad \textbf{(Eq. 10-13)}$$

Plug Eq. 10-12 and Eq. 10-13 into Eq. 10-8:

$$x = Y_{x/s}\left(s_0 - \frac{DK_s}{\mu_m - D}\right) \quad \textbf{(Eq. 10-14)}$$

K_s are all fixed. We specify the feed substrate concentration, s_o, and the operating dilution rate, D. The system of equations then leaves only x and s as unknown.

Since the system has two linear equations (x and s do not multiply to themselves or to each other) and two unknowns, it has a unique answer. Once D and s_o are specified, the solution of x and s can be determined. In other words, if one uses the same cell dilution rate and feed concentration, the culture will always reach the same steady state (Figure 10.2b). With the assumptions of Monod growth kinetics and constant yield, the model predicts a relatively constant cell concentration over an increasing D until D approaches the maximum specific growth rate (and the critical dilution rate, D_c), whereupon it begins to decrease. It can be further shown that the steady state is stable if the system is subjected to small fluctuations in feed concentration or dilution rate, and the cell and substrate concentrations return to the same steady state after fluctuation subsides. Although the experimentally observed kinetic behavior differs somewhat from the model prediction,[2, 3] this model of cell growth in a continuous reactor still serves the purpose of exploring various process behaviors, especially regarding experiments that are difficult to perform.

Under a given set of operating conditions, a continuous culture may have more than one steady state or none at all. A simple system such as the one shown in Figure 10.2 has unique steady states. In some cases, non-linearity may arise from a more complex relationship between substrate concentration and growth rate, or if the conversion yield (i.e., metabolism) is dependent on the substrate level. In those cases, the system of equations may have more than one feasible (non-negative) solution under some conditions. For such a system, multiple steady states may be observed; in other words, in two systems with identical dilution rates and feed concentrations, the steady state reached may differ depending on the trajectory of the culture. This has been seen in many studies due to the complexity of metabolism in mammalian cells. For a system that has multiple steady states, one needs to control the culture condition to follow a specific trajectory to the target steady state.[4, 5]

Continuous Culture with Cell Recycle

The development of continuous cultures for the manufacturing of protein biologics was driven by the production of recombinant proteins that are labile and produced at low levels. The short product holding time that can be attained in a continuous culture is necessary for their production. However, a simple continuous culture is hindered by a low cell concentration and low productivity. In principle, increasing the substrate concentration in the feed, as shown in Eq. 10-8, can increase cell concentration. However, the correspondingly increased metabolite concentration (see Eq. 10-10) will lead to growth inhibition. To overcome this constraint, cell recycling is employed.

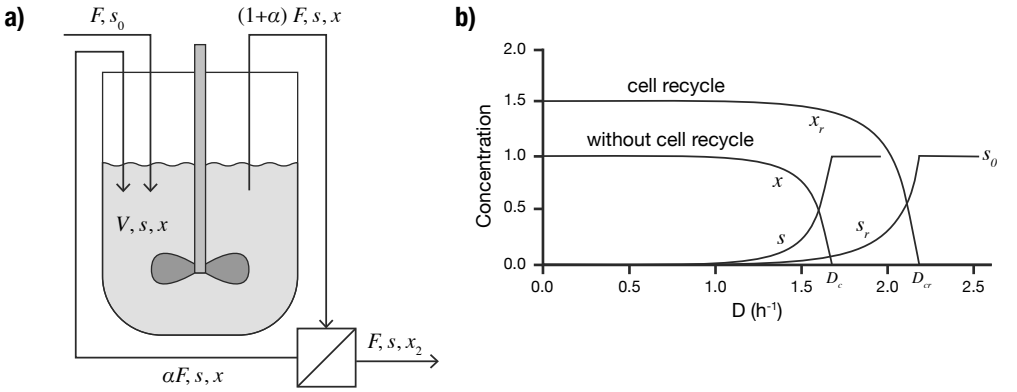

Figure 10.3. (a) A continuous culture with cell recycle. (b) Steady state cell and substrate concentrations at different dilution rates with and without cell recycle.

Panel 10.4. Continuous Culture with Cell Recycle

Balance on biomass for the reactor is

$$V\frac{dx}{dt} = \alpha Fcx - (1+\alpha)Fx + \mu xV \qquad \textbf{(Eq. 10-15)}$$

Balance on the cell recycle system gives

$$(1+\alpha)Fx = \alpha Fcx + Fx_2 \qquad \textbf{(Eq. 10-16)}$$

$$\frac{x_2}{x} = 1 + \alpha - \alpha c \qquad \textbf{(Eq. 10-17)}$$

The balance on the substrate is

$$V\frac{ds}{dt} = Fs_0 - \frac{\mu x}{Y_{x/s}}V - F(1+\alpha)s + \alpha Fs \qquad \textbf{(Eq. 10-18)}$$

Defining $F/V = D$

$$\frac{\mu}{D} = 1 + \alpha - \alpha c \qquad \textbf{(Eq. 10-19)}$$

$c>1$, so $D>\mu$

At steady state,

$$0 = D(s_0 - s) - q_s x \qquad \textbf{(Eq. 10-20)}$$

$$x = \frac{D(s_0 - s)}{q_s} \qquad \textbf{(Eq. 10-21)}$$

A general system of continuous culture with cell recycle is shown in Figure 10.3a. The reactor volume is kept constant by having the same flow rate of fresh medium entering the reactor as there is culture content exiting it. The fresh medium stream is free of cells, and the effluent stream has the same cell and nutrient (substrate) concentrations as in the reactor. A cell separator divides the effluent stream into a concentrated cell stream (with a cell concentration of c_x and a flow rate of αF) that is recycled back into the reactor and a low cell density stream that is purged. Note that the effluent stream from the reactor has a flow rate of $(1+\alpha)$ F, which is higher than the fresh feed. At steady state, the recycle stream has a flow rate of αF.

A material balance can be performed on the reactor and the cell recovery/recycling device for both cells and substrate in order to obtain the relationship among key process variables (Eqs. 10-15 to 10-21, Panel 10.4). The dilution rate is defined as the flow rate into the system (not the combined flow rate with the recycle stream into the reactor) divided by the reactor volume. Note that the relationship between biomass and substrate (Eqs. 10-20 and 10-21) is identical to that without cell recycle (Eqs. 10-7 and 10-8). However, with cell recycle, D and x are both higher than the corresponding specific growth rate and substrate concentration without cell recycle. By assuming Monod growth kinetics, one can plot out the steady state concentration profile as a function of the dilution rate (Figure 10.3b). With cell recycle, the dilution rate is higher than the specific growth (Eqs. 10-22 and 10-23, Panel 10.5). Furthermore, the cell concentration in the reactor is higher and the washout dilution rate is extended beyond the maximum growth rate (Eqs. 10-24 and 10-25, Panel 10.5). The enhanced performance of the system, or the extent of increase in the dilution rate from the specific growth rate, is described by the enhancement factor ϕ. Its value is affected by the recycling factor, α, and the cell concentration factor, c, as related by Eq. 10-22.

Panel 10.5. The Critical Dilution Rate of a Continuous Culture with Cell Recycle

Let $\phi = \dfrac{1}{1+\alpha-\alpha c}$, **(Eq. 10-22)**

$$\phi\mu = D \qquad \textbf{(Eq. 10-23)}$$

where ϕ = recycle enhancement factor

$$\phi\frac{\mu_m s_0}{K_s + s_0} = D_c \qquad \textbf{(Eq. 10-24)}$$

$$D_c \cong \mu_m \cdot \phi \qquad \textbf{(Eq. 10-25)}$$

$\therefore D_c > \mu_m$ for a cell recycle system, thus allowing a dilution rate higher than μ_m to be used.

Effects of a recycling factor

In a simple continuous culture, once the Monod model relationship between the specific growth rate and the substrate concentration is imposed (fixing K_s and μ_m), and the operating conditions of feed concentration (s_0) and dilution rate (D) are fixed, the steady state cell and substrate

concentration for a given dilution rate is also fixed. This is because a system of two linear equations (Eqs. 10-13 and 10-14) gives only one unique set of s and x. In a system with cell recycle, the system of equations (Eqs. 10-19 and 10-21) has α and c as parameters in addition to x and s. The system will settle to a prescribed steady state when both α and c are specified. A perfusion culture inherently has more degrees of freedom than a simple continuous culture. To ensure that the same steady state is reached consistently, more operating parameters must be fixed.

The key factor that cell recycle introduces that increases productivity is the amount of biomass ($aFcx$ in Eq. 10-16) returned to the reactor. To return the same amount of biomass, one may use a less concentrated cell stream (lower cx) and a higher flow rate (larger αF) or a more concentrated cell stream with a lower flow rate. In Figure 10.4, each line represents a steady state that has the same dilution rate and cell concentration, which has been achieved by a different combination of recirculation flow rates (αF) and different degrees of concentration factor c. A low recycling rate (αF) can be employed by using a highly efficient cell separator with a concentrated recycle stream. Conversely, when using an inefficient cell separator that gives a low degree of cell concentration, a large recycling rate needs to be used. In other words, cells will need to be pumped out of the reactor and passed through the cell separator to be recycled more frequently.

In large-scale operations, the cell stream could potentially exist outside of the reactor for a prolonged time, so oxygen starvation is a concern. Consequently, the fluid stream out of the reactor is often chilled to reduce oxygen consumption. With a device that gives a low c value, cells will need to be subjected to more frequent environmental perturbations, such as going through the pump and being chilled. This factor should be considered when selecting a cell recovery method.

Perfusion Culture with an Independent Cell Purging Stream

Some cell recycling devices are sensitive to fluctuations of operating parameters, and return the recycling cell stream with somewhat varying cell concentration. The cell concentration factor, c, is also affected by the chemical and physical environment. As c changes, the recycling flow rate must also change in order to maintain a steady state. Increasingly more perfusion cultures employ an internal or external microfiltration membrane to withdraw a cell-free stream while using another purge stream to remove some cell mass to maintain a steady state. The cell purge rate is used as a means of controlling cell concentration at a steady value.

The material balance equations for a system (Figure 10.5) that also considers the accumulation of growth-inhibitory metabolites are shown in Panel 10.6 (Eqs. 10-26, 10-27, and 10-28). The steady state

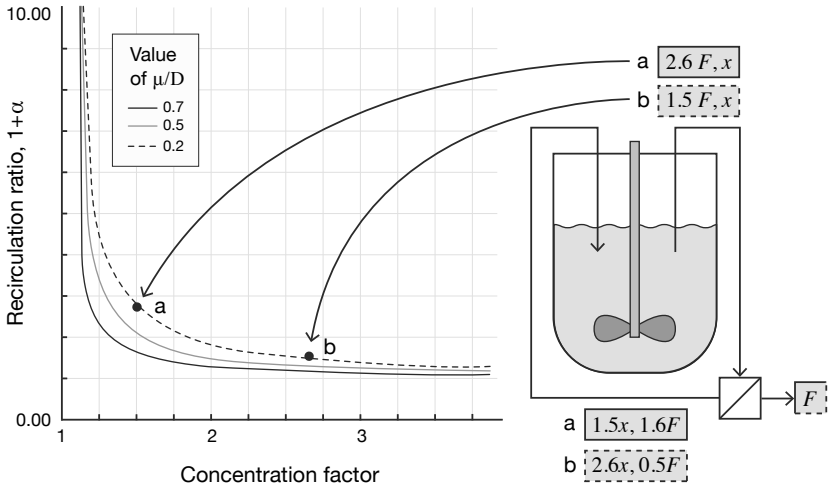

Figure 10.4. The relationship between the recirculation ratio and the cell concentration factor in a continuous culture with cell recycle. Two different recycler operating conditions (a and b) give the same cell concentration in the bioreactor at the same dilution rate.

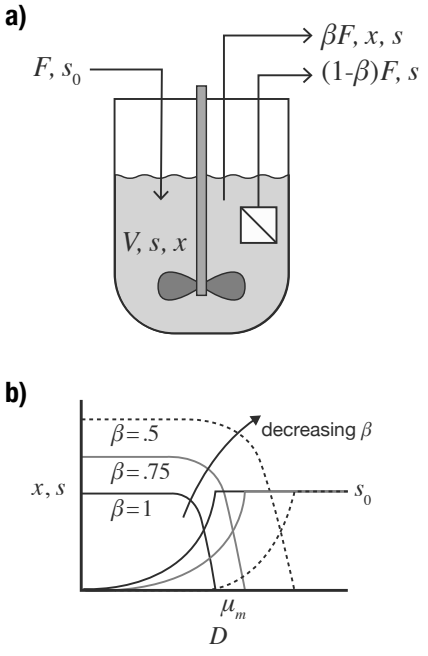

Figure 10.5. (a) A perfusion culture with a total cell retention device and cell purging. (b) Corresponding steady state profiles of cell and substrate concentrations.

Panel 10.6. Continuous Culture with Cell Retention and a Purge Stream

$$\frac{dx}{dt} = \mu x - \beta D x \qquad \textbf{(Eq. 10-26)}$$

$$0 < \beta \leq 1$$

$$\frac{ds}{dt} = D\left(s_0 - s\right) - q_s x \qquad \textbf{(Eq. 10-27)}$$

$$\frac{dI}{dt} = \alpha_{I/s} q_s x - DI \qquad \textbf{(Eq. 10-28)}$$

At steady state

$$\mu = \beta D \qquad \textbf{(Eq. 10-29)}$$

$$x = \frac{D\left(s_0 - s\right)}{q_s} \qquad \textbf{(Eq. 10-30)}$$

$$I = \frac{\alpha_{I/s} q_s x}{D} \qquad \textbf{(Eq. 10-31)}$$

concentrations of cells and substrate for Monod growth kinetics are shown in Figure 10.5b. The dilution rate is higher than the growth rate, as shown in Eq. 10-28; D is always larger than μ since $\beta < 1$. The system does not have a steady state if there is no purge stream (i.e., $\beta = 0$ in Eq. 10-26) while the growth rate is not zero. Cells in suspension generally do not remain in a static, non-growing state over a long period. Thus, a purge stream that withdraws cells continuously or periodically is necessary. Cell retention increases the cell concentration in the reactor, as seen in the previous case of using an external cell recycling device. Such a complete cell retention device (for example, microfiltration, discussed later in this chapter) gives the system only one more degree of freedom in its operation compared to a simple continuous culture. For a system where the relationship between the growth rate and substrate concentration behaves like Monod kinetics, as long as the purge rate (βF), feed substrate concentration, and dilution rate are fixed, the steady state is also determined. We also noted that the balance equation between cell concentration and substrate concentration (Eq. 10-30) remains unchanged from the other cases discussed (Eqs. 10-8 and 10-21).

In a stirred tank bioreactor with cell retention or cell recycle, the inhibitor concentration can be kept at a low level to allow for a high feed concentration, which can subsequently support a high cell concentration (Eq. 10-31). By increasing only the medium components that are consumed by cells and balancing the feed concentration and the consumption rate, excess accumulation of unconsumed substrates and an increase in osmolality can be avoided. Such a stoichiometric balancing strategy is applied to both steady states and the transient stage of a ramping-up state designed to bring the cell concentration up to the target steady state level. It should be noted that overfeeding a fortified medium may also lead to an inhibitory accumulation of substrates, especially during the start-up transient period. Prior to the implementation of a perfusion culture, one may use an empirically derived relationship between the growth rate, lactate, and osmolality to simulate the kinetic behavior of the culture and optimize the feeding strategy.[6]

Perfused Fed-Batch Culture

When employing a perfusion culture, one aims for a long period (up to months) of steady state operation. In order to sustain a culture for a long period, cells are nourished to grow at a moderate rate. Another mode of operation is the employment of perfusion only to sustain a fed-batch culture, thereby enhancing its productivity by reaching a very high cell concentration and then extending the period that the reactor is at that concentration. This method is thus a combination of the fed-batch culture discussed in Chapter 9 and the perfusion culture described previously in this chapter. The stoichiometric principle of balancing the feed

with the cells' demand still applies. Overall, a perfusion or perfused fed-batch culture is performed in a small-scale reactor, often a disposable one. In contrast, fed-batch cultures can be operated on a scale of tens of thousands of liters.

The balance equations on cells, product, substrate, and metabolites are shown in Panel 10.7. A perfused fed-batch culture may undergo a number of stages: initial start-up, intermittent feeding, perfused growth rate, perfused stationary, and decline (Figure 10.6). The balance equations are the same for all of the stages, but the values of the parameters for those equations can vary widely. For example, in the biomass equation the discharge term is removed and a cell death term is added, since cell death is prominent at the last stage (Eq. 10-32, Figure 10.6). A balance equation is also added for the product (Eq. 10-33), since the vast majority of the product is most likely produced in the perfusion stages. The specific product formation rate is strongly affected by the chemical environment. Note that in this case, the system is not operated at a steady state, because it does not have cell purging; the viability and productivity are therefore likely decreasing. In some cases, the cell concentration is so high that even at the full capacity of the cell recycle device a continued accumulation of metabolites is unavoidable. The optimization effort for a perfused fed-batch culture may thus focus on identifying the chemical environment that gives the highest productivity and the longest production period.

Panel 10.7. Material Balance on a Perfused Fed-Batch System

$$\frac{dx}{dt} = \mu x - \mu_d x \qquad \textbf{(Eq. 10-32)}$$

$$\frac{dP}{dt} = q_p x - DP \qquad \textbf{(Eq. 10-33)}$$

$$\frac{ds}{dt} = D(s_0 - s) - q_s x \qquad \textbf{(Eq. 10-27)}$$

$$\frac{dI}{dt} = \alpha_{I/s} q_s x - DI \qquad \textbf{(Eq. 10-34)}$$

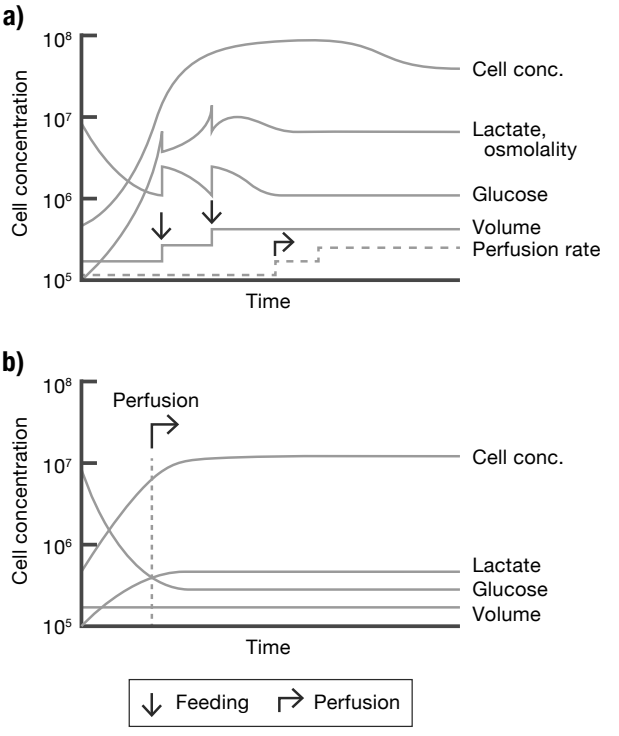

Figure 10.6. Kinetic behavior of (a) a hybrid perfused fed-batch culture and (b) a perfused culture.

Methods of Cell Retention

A cell retention device for perfusion needs to meet more stringent requirements than those for cell separation in downstream processing. It must maintain the aseptic condition for an extended period of time and be mechanically reliable or easily replaceable in a manufacturing setting. These devices give different ranges of the concentration factor (c), thus requiring different recycling factors (α) to achieve the same degree of recycle enhancement (ϕ) (Eq. 10-22). The device that gives a low concentration factor will have to circulate the culture fluid more often, i.e., require a larger flow rate out of the reactor ($(1+\alpha)F$).

Cell Separation Using Settling Velocity

Sedimentation

The simplest cell separator is perhaps the conical settler (Panel 10.8).[7] The cell stream from the bioreactor enters the settler in a region with a large cross-sectional area designed to reduce the flow velocity. In this region, the fluid flow separates into an upward and a downward stream. The flow rate of the upward stream is adjusted so that its vertical velocity is smaller than the cells' settling velocity (Figure 10.7). Hence, cells are separated from the upward stream and carried by the downward flow to the recycle stream that is returned to the reactor.

Panel 10.8. Conical Settler

- Selective removal of dead cells
- Low separation efficiency
  - Cell settling velocity ~2–10 cm/h
  - Good for large cells, aggregates, microcarriers
- Long residence time outside the bioreactor

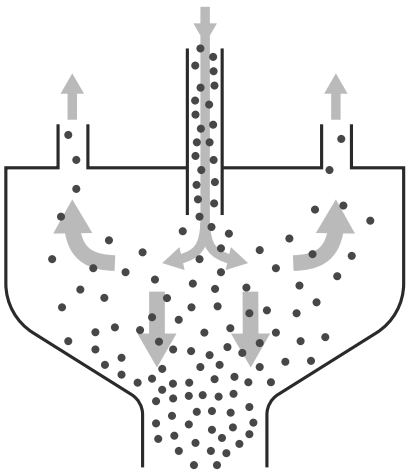

Figure 10.7. A settling cone for cell recycle.

It is important to note that the separation behavior is sensitive to the size of the settler and the scale of the reactor. As the reactor scale increases, the recycling flow rate also needs to increase proportionally. However, the cross-sectional area of the settler increases only with 2/3 the power of the settler volume. The efficiency of cell separation thus decreases as the scale increases. The method is particularly useful in separating large particles. Some industrial processes employ microcarriers with particle diameters ranging from 0.2 mm to 2 mm, which are separated from the medium stream via sedimentation. In some cases, cells are grown as large clumps, or aggregates, of 1–2 mm in size, thus making sedimentation readily applicable.

Inclined settling

In a simple settling tank, the direction of fluid flow and cell settling are along the same axis (both vertical). A sufficiently long transient zone is necessary to separate the cell and cell-free

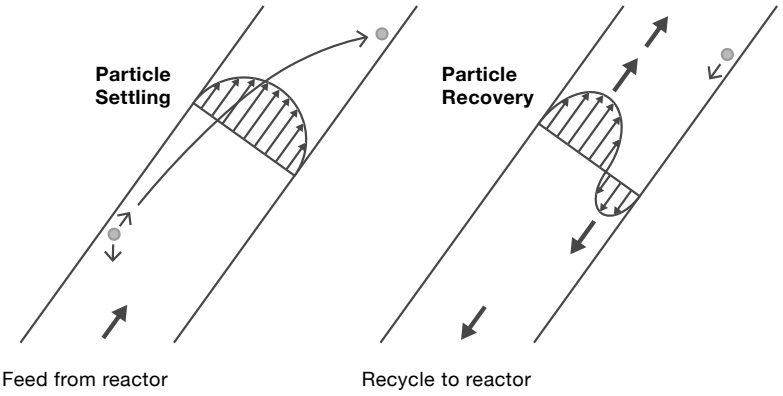

Figure 10.8. Cell separation in an inclined settler for cell recycle.

streams. To enhance the separation efficiency, the settler is often inclined so that the fluid flow direction and the particle settling direction are at an angle (Figure 10.8, Panel 10.9).[8] The feed cell stream enters at the bottom and moves upward. Inside the settler, cells begin to "settle" down vertically due to gravity. If a cell particle hits the surface of the lower plate, it is "collected," since the fluid velocity on the surface is zero. The effluent stream exiting the settler from the top carries fewer cells than when it enters. Eventually, the cells that settled on the bottom plate form a layer of fluid that has a higher density than the stream above. This heavy stream then moves downward, carrying the cells along with it. At a steady state, there are three streams in the system: the feed stream, the effluent stream (carrying unsettled cells), and the concentrated cell stream exiting at the bottom.

Panel 10.9. Inclined Settling

- While a particle is moving upwards with the flow, it also settles towards the bottom plate
- It is "collected" upon hitting the bottom
- Eventually, the particle-rich zone has a higher fluid density and begins to move downward
- The particle-rich stream is recycled to the bioreactor

In industrial design, multiple inclined plates are used in a single settler. In such designs, the feed stream and the returning cell stream are partitioned in different zones to avoid intersecting with each other. In some cases, mechanical vibration is applied to the plates to prevent settled cells from sticking to the surface and being lysed.

The residence time in the settler has to be at least as long as the particle settling time. With a high cell concentration in the stream, oxygen starvation is a major concern, as it may induce apoptosis and cell lysis. Therefore, the stream passing through the settler is often chilled to reduce the cells' metabolic rates.

Acoustic cell separator

To enhance settling, an acoustic resonance device was developed (Figure 10.9).[9] This device uses acoustic energy to enhance cell agglomeration.

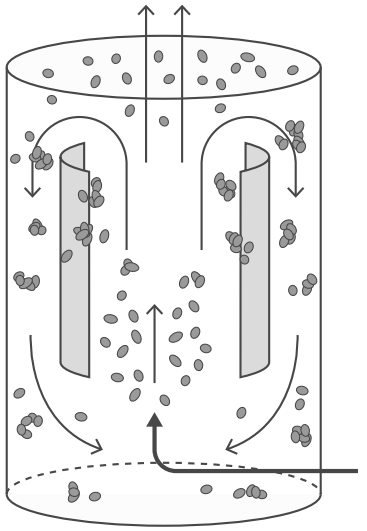

Figure 10.9. An acoustic cell agglomeration device for cell recycle.

Panel 10.10. Centrifugation

Centrifugation Technology

- Excellent separation efficiency
- High perfusion capacity
- Little clogging
- Easy scale-up
- Vulnerable to mechanical failure during long-term continuous operation

Different Designs

- Disk type
  - Continuous cell recycle back to fermenter
- Disposable separation unit

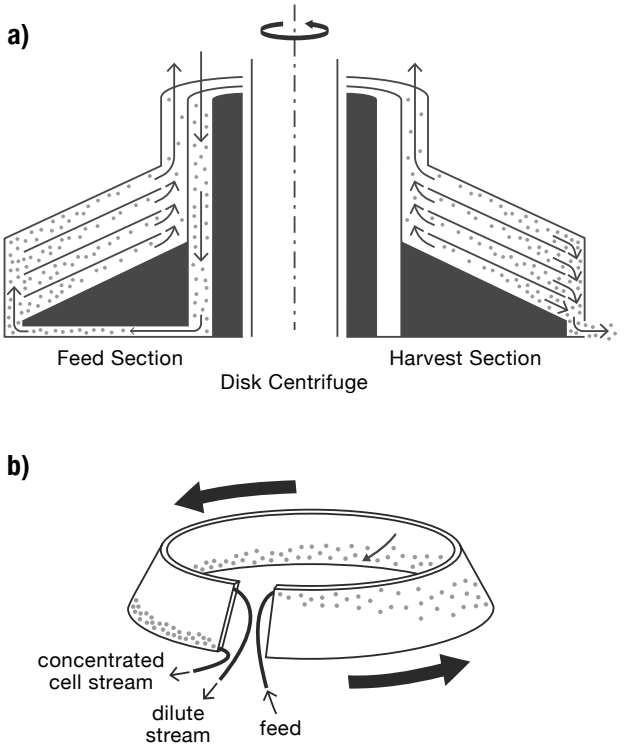

Figure 10.10. (a) A steam-sterilizable disk-type centrifuge. (b) A disposable bag for a Centritech centrifuge for continuous cell separation.

Cells are induced to agglomerate as they pass through the acoustic chamber, giving rise to a faster settling velocity. With an increased settling velocity, sedimentation is easily accomplished without resorting to a large settling device with a long settling time.

Centrifugation

Centrifugation is a standard unit operation in many downstream recovery processes (Panel 10.10). However, most centrifuges are not designed for long-term continuous and aseptic operations. In the early stages of perfusion culture development, in-line centrifuges were used for intermittent cell recycling. They were also used to remove serum-containing spent medium and replenish it with fresh medium for production. As a result, a number of steam-sterilizable centrifuges were subsequently developed. These centrifuges and the disposable bag-based centrifuges are all capable of processing up to hundreds of liters of medium a day and are used in perfusion culture (Figure 10.10). The disk-type centrifuge is analogous to a multiple parallel plate settler, except that the parallel plates are rotating and generating a centrifugal field for cell settling. The disposable bag system employs three tubes: a feed tube, an outflow tube for the heavy (cell-rich) stream, and an outflow tube for the light stream.[10] The unique design of an inverted question mark allows the three tubes to rotate along with the centrifuge without becoming twisted.

Centrifugal-Force-Enhanced Cell Sieves

Spin filter: Centrifugal filter and rotating cage

A spin filter device is a cylindrical rotating cage with a high porosity filter with relatively large openings (~20–100 μm) installed on the inner wall. The whole device is submerged in the culture fluid (Figure 10.11). A pump draws medium from inside the cage as the purge stream. The culture fluid passes through the filter and enters the cage, though some cells are excluded and remain outside. Thus, the cell concentration inside the cage is lower than that in the bulk culture fluid which achieves the overall retention of cells in the reactor. The cage rotates along the center shaft of the impeller agitator at a low speed. The

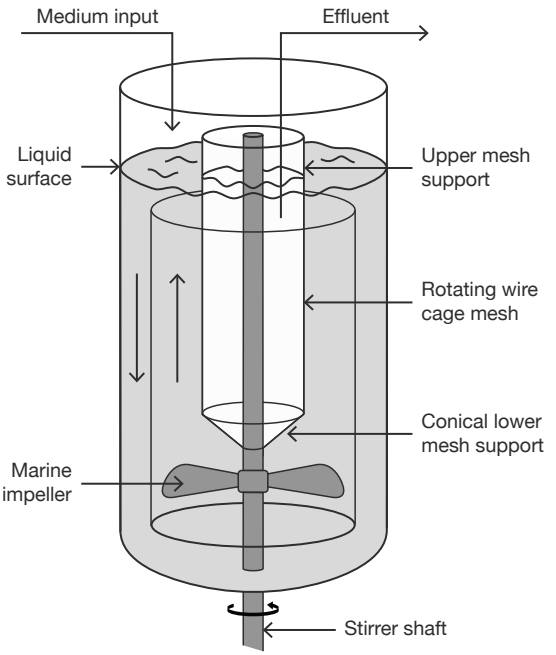

Figure 10.11. A spin filter bioreactor. The culture fluid for effluent flow is withdrawn from inside the wire cage.

centrifugal field is typically insufficient to push cells away along the outside wall of the cage. It is plausible that the liquid layer around the cage has a lower cell concentration than in the bulk. As a result, the fluid drawn across the filter has a lower cell concentration than in the bulk. The system has been employed in moderate scales of up to hundreds of liters.

The rotating cage is difficult to scale up, as its operating mechanism is not well understood. Later modifications of the spin filter increased its rotation rate up to hundreds of rpm, thus allowing it to operate like a centrifugal filter. The centrifugal force pushes the cells away from the surface of the filter, thus drawing liquid through at a lower cell concentration than that in the bulk. In some variations, the centrifugal filter is installed outside of the reactor and used as an external cell retention device.

Membrane Separation

Microfiltration and alternating tangential filtration

Microfiltration uses membranes of different configurations, including parallel plates and hollow fiber devices, which have a pore size of around 2–4 μm. Microfiltration was among the first techniques used for cell retention. Its widespread use was impeded by membrane fouling, which is especially severe when a complex medium with a high concentration of proteins is used in the culture or when cell viability is low. With the increased use of low protein medium in the last decade, the problem of protein fouling has lessened, but the clogging by debris from dead cells remains problematic. Most microfiltration devices used for perfusion culture are of the tangential flow type; as the fluid passes through the fiber, a small portion permeates through the fiber and the cell-free permeate is collected in the extracapillary space and discharged. Meanwhile, the cell-containing retentate stream is returned to the reactor.

Microfiltration devices are configured for tangential flow, meaning that the feed stream flows in a direction parallel to the membrane while the filtrate flows across it. The more recent use of a pulsatile flow system, often called alternating tangential filtration (ATF), uses a diaphragm pump to periodically reverse the flow direction. This generates a transmembrane pressure difference which pushes the medium filtrate out while retaining cells in the lumen side of the device, which is returned to the reactor (Figure 10.12).

ATF has become commonly used in perfusion and hybrid perfused fed-batch culture in the past few years.[1] Detailed analysis of its operating parameters has not yet emerged. For example, the optimal range of the Reynolds number for luminal flow and transmembrane pressure drop, as well as its effect on membrane fouling, have not been reported. The transmembrane pressure drop drives the filtrate flux. At a high cell concentration, a high transmembrane flux may carry cells toward the membrane pores and cause damage. A low transmembrane flux will require a higher recirculation rate (i.e., the number of times the culture fluid

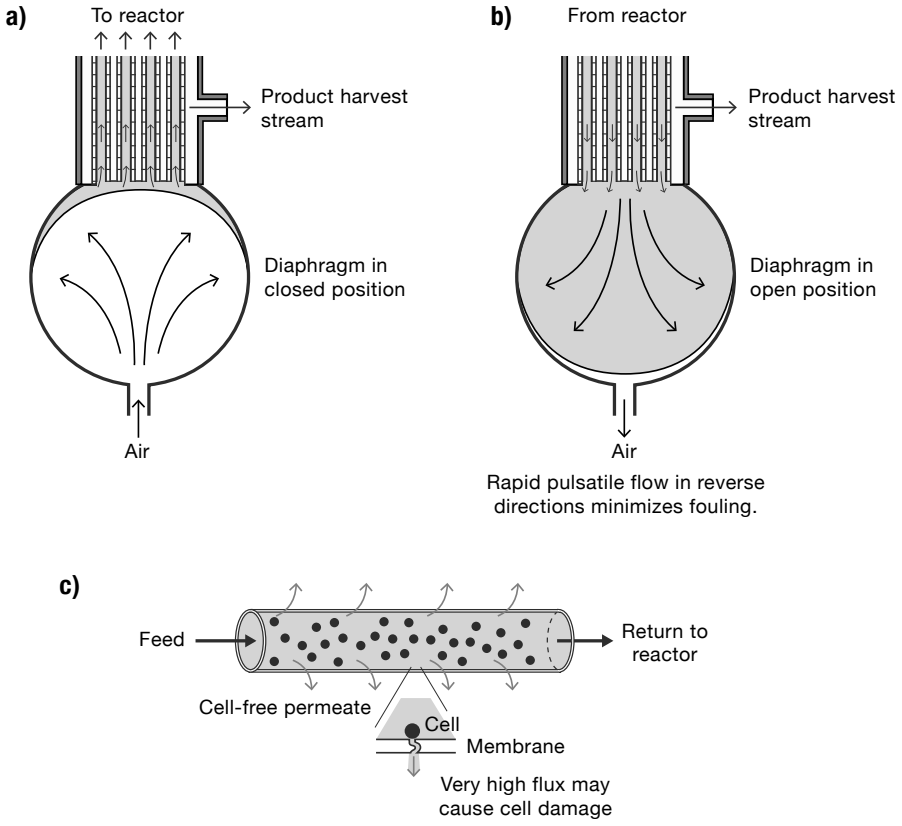

Figure 10.12. (a, b) A tangential flow hollow fiber filter device in its pumping cycle. (c) A depiction of transmembrane flux.

passes through the device each day) to achieve the same perfusion rate; in turn, this may result in more frequent exposures to high shear stress regions near the entrance to the membrane. Given that the Reynolds number in blood flow ranges from a very small number in the capillaries to a few thousand in the highly turbulent regions of the arteries, neither the flow in the fiber lumen nor the entry into the reservoir area are likely to cause severe cell damage. Nevertheless, careful analysis should be undertaken in developing processes using a membrane cell retention device.

Both membrane- and settling-based cell retention methods face challenges in scaling up. The capacity of a settling device is dependent on the surface area perpendicular to the direction of settling for cell collection, while the capacity of a membrane separator is dictated by the membrane area available for the permeate to pass through. As the volumetric flow rate of perfusion increases, the area required for cell separation increases proportionally. Increased transmembrane flux can somewhat increase the capacity, at the expense of increased membrane fouling and potential cell damage. In some cases, multiple units of ATF are used to meet the demand of removing metabolites.

Continuous Culture & Continuous Process

With the use of a membrane system for cell retention, the effluent stream is cell-free. This allows for the isolation or capture of a continuous product. As illustrated in Chapter 1, a typical cell culture product recovery process involves cell removal using membrane separation or centrifugation. Product isolation entails adsorption chromatography to reduce the process volume, followed by product purification by ion exchange, hydrophobic interaction chromatography or other chromatography, virus inactivation/filtration, and final polishing (for an overview, see Chapter 13 in *Engineering Principles in Biotechnology* (reference [11])). Since the effluent stream from the bioreactor is cell-free, it eliminates the need for cell removal. By operating at a very high cell density, the resulting product concentration may reach a level that does not require further concentration using ultrafiltration before feeding into an adsorption column such as a protein A chromatography. This enables almost direct feeding from the bioreactor into the downstream process, barring a medium particulate removal step and perhaps a buffer exchanging diafiltration step.

There have also been attempts to make the product capture adsorption step continuous. This is usually done by using a split-column strategy.[1] As an illustration, we consider a column that is operated with a cycle of three stages: feed-stream loading, washing, and elution (Figure 10.13). The three stages have an equal time interval of θ. Now we split the column into three sections. We can use the same flow rate of feed, washing, and elution stream as before, with the first column being loaded with feed stream while the second and third columns are being washed and eluted. Since the capacity of each column is only one-third of the original volume, after a period of $1/3\theta$ the first column will be fully loaded, the second washed, and the third eluted. The feed stream is then switched to load the third column, while the first column is washed and the second eluted. In the original single-column operation, a complete cycling time is 3θ, but the feed flow is only on for a period of θ. In the split-column fashion, the feed stream is being continuously fed into one of the three columns at the same flow rate as before. This method bears much similarity to moving-bed chromatography. The split-column method also has an additional advantage because it avoids unadsorbed product loss due to column breakthrough. In the adsorption process, it is inevitable to have a breakthrough, because at the end of the adsorption the flow pattern in the front is not like a piston flow, but rather a gradient decrease. To avoid product loss, feeding is stopped as soon as the unadsorbed product begins to elute at the end of the column. This reduces the column

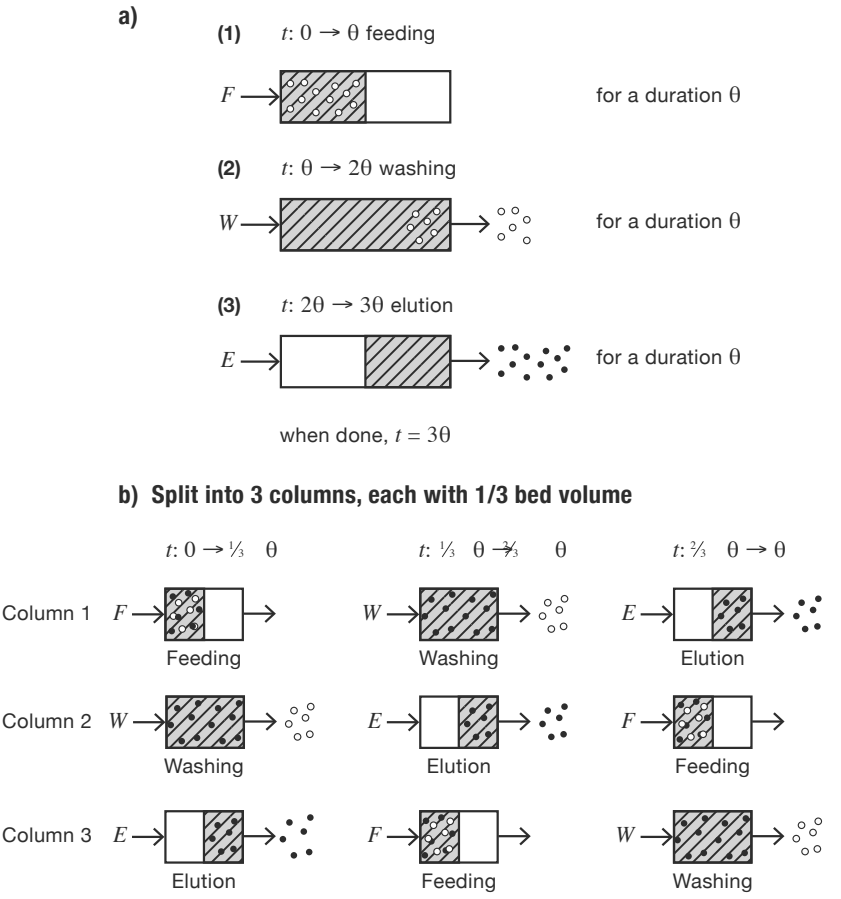

Figure 10.13. Simulated continuous adsorption chromatography. A hypothetical three-stage split column is shown cycling through the feeding (adsorption), washing, and elution stages.

utilization efficiency. Alternatively, one can use the entire column capacity by letting the product elute until the column is fully loaded. But in that case, the unadsorbed product is lost or has to be collected and loaded again in the next adsorption process—an option that is not available if each run is a separate product lot, since different lots of material cannot be mixed. Using the split-column method, the unadsorbed product is directed to the second column and is not lost.

The control of column switching may not be as complicated as in moving-bed chromatography, but it still requires extensive optimization. The product capture step reduces much of the process volume. There are efforts to make the downstream unit operations even more continuous, though their use of smaller process volumes may mean that a conversion to a continuous process would not yield much benefit.

Concluding Remarks

Cell growth in a bioreactor is limited by the accumulation of many inhibitory metabolites in the culture. Unlike our bodies, which have multiple organs working to convert the metabolites, balance the nutrients, and excrete the remaining metabolites, cells in culture lack these capabilities. Fed-batch culture has been the prevailing mode of cell culture processing in the production of recombinant proteins. Continuous culture, traditionally used mostly for the production of labile products, is increasingly being adopted to prolong cultivation periods and increase productivity. The increased adoption of perfusion has been facilitated by advances in cell retention technology and the development of less complex media. This has also led to an increased adoption of perfused fed-batch culture.

A very important design parameter for both fed-batch and continuous culture is the feeding strategy. Foremost, the feed medium, usually with a fortified nutrient composition, should be stoichiometrically balanced to minimize the accumulation of excess nutrients and inhibitory metabolites, and to reduce the increase of osmolality. It is likely that perfusion will be increasingly adopted for moderate-scale processes, especially those employing disposable bioreactors, whereas very large-scale fixed-tank processes will continue to operate in fed-batch mode. It is important to remember that a system will robustly reach a steady state only when the operation variables are sufficiently specified such that the system of equations describing the culture has solution(s) at a steady state. For a perfusion culture, the number of operation variables is greater than that for a simple continuous culture, and one must specify more operation variables in order to make the process robust. Given the intrinsic advantages of continuous operation and the advances in cell retention technology, we may begin to see a more widespread application of perfusion culture in the coming years. It has considerable potential to increase the capacity of high-throughput processes, reduce reactor sizes, and possibly minimize product quality fluctuations throughout steady state operations.

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