CELL CULTURE TECHNOLOGY

1. Introduction

Cell culture processes, the *in vitro* growth of animal, insect, or plant cells on a large scale to manufacture biochemicals of commercial importance, have been used for some time for the manufacture of viral vaccines (see VACCINE TECHNOLOGY). Significant growth in this technology, primarily because of the advent of recombinant DNA methods (see GENETIC ENGINEERING) for the production of therapeutic proteins (qv) and hybridoma technology for production of monoclonal antibodies (see IMMUNOASSAY) occurred in the late 1980s and 1990s. The need for cell culture technology stems mainly from the fact that bacteria do not have the capability to perform many of the posttranslational modifications that most large proteins require for *in vivo* biological activity. These modifications include intracellular processing steps such as protein folding, disulfide linkages, glycosylation, and carboxylation.

Historically, large-scale cell culture technology traces its roots to the production of viral vaccines and other therapeutically important secreted products of human and primate cells such as those listed in Table 1. Most of these products were made by cells grown either in suspension or attached to microcarriers. Such cells were cultured in stirred tank reactors adapted from bacterial fermentation (qv) processes. The primary challenges in adapting microbial fermenters to the rigors of cell culture technology were reducing the risk of microbial contamination, modifying the agitation system to provide low shear agitation to the shear-sensitive mammalian cells, and providing adequate oxygenation under the low shear conditions mandated by the cells (see AERATION, BIOTECHNOLOGY).

The major applications for cell culture technology are found in the production of Monoclonal Antibodies (MAbs), recombinant therapeutics and vaccines. Monoclonal antibodies were initially used in small quantities for diagnostic purposes. However, in the past few years several monoclonals have been developed for therapeutic purposes. The first therapeutic Mab (OKT-3) was developed by Johnson & Johnson to prevent rejection of organ transplants. This was produced in mouse ascites as the requirements were relatively small. Several new Mabs have been introduced in the market, that utilize suspension culture technology. For example, Johnson & Johnson has introduced ReoPro for prevention of blood clotting during high risk angioplasty. More recently, IDEC Pharmaceuticals

Table 1. **Historical Products of Cell Culture Technology**

Product	Cell line	Process[a]	Year introduced	Reference
FMD[b] vaccine	baby hamster kidney	SC	1962	1
rabies vaccine	dog kidney	SM	1978	2
interferon	human namalwa	SC	1979	3
polio vaccine	monkey kidney	SM	1980	4

[a] suspension culture = SC; stirred microcarrier = SM.
[b] Foot-and-mouth disease = FMD.

Table 2. **Examples of Therapeutic Products Manufactured by Cell Culture**

Product	Company	Therapeutic use
OKT-3	Johnson & Johnson	prevention of transplant rejection
Zenapax	Hoffmann	prevention of transplant rejection
ReoPro	Johnson & Johnson	antiplatelet for high risk angioplasty
Rituxan	Idec Pharmaceuticals	non-Hodgkin's lymphoma
Remicade	Johnson & Johnson	rheumatoid arthritis
Herceptin	Genentech	breast cancer
Avakine	Johnson & Johnson	Crohn's disease
Synagis	Medimmune	RSV
Activase	Genentech	heart attacks, strokes
Epogen	Amgen	anemia in kidney dialysis patients
Aranesp	Amgen	anemia in kidney dialysis and cancer chemotherapy patients
Kogenate	Miles/Cutter	hemophelia
Pulmozyme	Genentech	cystic fibrosis
Avonex	Biogen	multiple sclerosis
Enbrel	Amgen	rheumatoid arthritis
Mylotarg	Celltech Chiroscience	acute myeloid leukemia
Zevalin	Idec Pharmaceuticals	non-Hodgkin's lymphoma
Xolair	Genentech	allergic asthma
Humira	Abbott	rheumatoid arthritis

received FDA approval for Rituxan for the treatment of non-Hodgkins lymphoma, Hoffmann La Roche received approval for Zenapax—A humanized monoclonal to prevent transplant rejection, Genentech received approval for Herceptin (for treatment of metastatic breast cancer), and Medimmune received approval for Synagis (a treatment of respiratory synctial virus). Additionally, Johnson & Johnson introduced Avakine for the treatment of Crohn's disease. Some examples of these products are shown in Table 2 (along with the therapeutic Mabs mentioned earlier). Vaccines also continue to be produced by cell culture. A recent example of this is the vaccine against chicken pox (Varivax) introduced by Merck.

Finally, insect cell culture is being utilized increasingly for quickly producing research quantities of new proteins using the baculovirus expression system (5). The strong polyhedrin promoter and the insect's cells ability to perform many posttranslational modifications have made the system useful for the expression of mammalian proteins that cannot be produced in native form in *Escherichia coli*. Technology development for these products has centered around the differences in characteristics of mammalian versus microbial cells, notably, the shear sensitivity and susceptibility to contamination of the mammalian lines.

Although the focus of this article is mainly on mammalian cells, the technologies described herein also apply in principle to insect and plant cells.

2. Characteristics of Mammalian Cells

2.1. Environmental Conditions. Mammalian cells *in vivo* are maintained in a carefully balanced homeostatic environment and thus have evolved

Table 3. **Environmental Parameters for Mammalian Cell Cultivation**

Parameter	Range	Typical value
pH	6.6–7.6	7
temperature, °C	33–39	37
dissolved oxygen, Pa[a]	0.7–40	10
osmolarity, mOsm/kg[b]	280–360	300
dissolved CO_2, Pa[a]	0.9–20	7
tolerable shear rate, s^{-1}	0–3000	1500

[a]To convert Pa to mm Hg, multiply by 7.5.

[b]Milliosmolar or milliosmole = mOsm, where an osmole equals 1 mol of solute divided by the number of ions formed per molecule of the soluble, ie, 1 mol of sodium chloride is equivalent to 2 osmoles of sodium chloride and 1 *M* NaCl = 2 Osm NaCl.

to require fairly stringent environmental conditions. These cells differ significantly from bacterial cells in that they lack a rigid cell wall, and are hence much more shear sensitive. Many animal cells are also attachment dependent, needing a surface to grow on. Many of the cell culture technologies provide the low shear, high surface area environment needed for the mammalian cells. Another approach, however, is to adapt cells to suspension culture and select cells that are less shear sensitive permitting the use of fermentation technology for the culture of animal cells. The optimum environmental parameters depend on cell type and are specific to cell type. Typical ranges for some of these parameters are listed in Table 3.

2.2. Nutritional Requirements. The nutrient requirements of mammalian cells are many, varied, and complex. In addition to typical metabolic requirements such as sugars, amino acids (qv), vitamins (qv), and minerals, cells also need growth factors and other proteins. Some of the proteins are not consumed, but play a catalytic role in the cell growth process. Historically, fetal calf serum of 1–20 vol% of the medium has been used as a rich source of all these complex protein requirements. However, the composition of serum varies from lot to lot, introducing significant variability in manufacture of products from the mammalian cells.

Serum is expensive, the 2003 price is ~$470/L, and supply depends on cattle supply. Use of this serum also poses significant difficulties in validating processes for absence of viral contamination. Hence, a goal in cell culture technology is to develop serum-free media for cell culture. Much work has gone into developing serum-free media and a sizable portion of cell culture research is devoted to this project. Several recent publications have reported development of protein-free and animal component free media for cell culture (6–8).

Several generic media formulations have been developed for growth and cultivation of mammalian cells and are commercially available. Each contains amino acids, inorganic salts for providing the right osmolarity, essential minerals, and buffering capacity; vitamins, and energy sources such as glucose. Whereas mixtures of the different formulations are often used to optimize growth and productivity of cell lines, many of the basal media need to be supplemented with serum or other appropriate proteins (or other protein free components such as soy peptone or meat digests) for promoting cell growth. Some of the more commonly used media formulations include: minimum essential medium (MEM) for

a broad spectrum of mammalian cells; basal media eagle (BME) for diploid or primary mammalian cells; Dulbecco's modified eagle media (DMEM) for a broad spectrum of cells; CMRL media for Earl's "L" cells and monkey kidney cells; Fischer's media for murine leukemic cells; Iscove's modified Dulbecco's media (IMDM) for rapidly proliferating high density cell cultures; McCoy's media for human lymphocytes; Ham's F10 and F12 for Chinese hamster ovary cells and other mammalian cells; and RPMI 1630/1640 for suspension cells and human leukemic cells. These media are available from Gibco Laboratories (Grand Island, New York), Irvine Scientific (Santa Ana, California), Sigma Chemical Co. (St. Louis, Missouri), Hyclone Laboratories (Logan, Utah), and JRH (Lenexa, Kansas) among others. Most of these companies also offer proprietary serum free and protein free formulations for CHO, hybridoma, and other mammalian cells (eg, Ex-Cell brand from JRH Biosciences).

Another essential nutrient not supplied with the media is oxygen. The oxygen consumption rate of mammalian cell cultures is much lower than that of bacterial ones because cell densities are much lower than those achieved in bacterial cultures. The oxygen consumption rate varies from cell line to cell line, but the range has been reported to be as wide as 0.05–0.5 mmol/(10^9cells · h) (9). Hence, designing oxygenation systems for mammalian cells is a function of the cells being used. Use of the worst case scenario may lead to costly overdesign. This is especially so if silicone tube oxygenators are being considered. Direct sparging in the reactor to accomplish oxygen transfer often leads to cell damage unless protective agents, such as pluronic polyols, are used (10). However, as long as the pluronic is nontoxic to the cells and is compatible with downstream processing steps, this use is probably the most efficient route to oxygenating cell culture systems. In some cases, oxygenation via sparging can be used without significant damage to cells (and without the use of surfactants) as long as the sparging is resorted to only on demand. Most cell lines also require a small amount of dissolved carbon dioxide for growth, especially at low cell densities. However, at higher cell densities, carbon dioxide may build up in the bioreactors and impact product formation in negative ways. Recently, strategies for carbon dioxide removal from large scale fed batch cultures have been described (11).

2.3. Kinetics of Cell Growth and Product Formation. Mammalian cells grow at a much slower rate than bacterial and yeast cells (see YEASTS). The maximum specific growth rates for mammalian cells range from 0.01 to 0.05 h^{-1}, corresponding to cell doubling times of 14–70 h depending on the cell line and environmental conditions. Most primary cell lines are anchorage dependent and need a surface to grow on. They are also contact inhibited, ie, they stop growing once the surface is confluent. Alternatively, most of the cell lines used industrially for recombinant products and monoclonal antibodies are not attachment dependent. For example Chinese hamster ovary (CHO) cells are commonly used as host cells for recombinant products. These cells are transformed by tumor viruses and do not require a surface to grow on. They do, however, prefer to grow on surfaces and requirement for serum factors diminishes significantly when they are attached to surfaces. Thus CHO cells can be cultured for several weeks in protein-free media if grown on microcarriers, whereas they require serum proteins such as fetuin or appropriate protein substitutes (such as soy peptone) to grow in suspension. CHO cells can also be adapted to grow in

chemically defined serum-free and protein free media which are commercially available from many vendors. Most hybridomas used for making monoclonal antibodies are attachment independent, grow in suspension, and have minimal requirement for serum proteins. It is necessary to adapt these cells to serum-free media for several days before they start growing well in these media.

Cell growth kinetics of mammalian cells can be described by the typical lag, exponential growth, then stationary and death phases. The exponential phase may be described adequately by a Monod type of kinetic model when the growth rate is much larger than the death rate. At low growth rates, it is necessary to include cell death kinetics to account for the lower viability and to predict the cell viability. Toward the end of the exponential culture, cells are also subject to growth inhibition from metabolic by-products such as lactate and ammonia. Hence, for continuous processes, a comprehensive model should contain terms for cell growth, based on the limiting substrate concentration, cell death, and inhibition kinetics.

Product formation kinetics in mammalian cells has been studied extensively for hybridomas. Some studies suggest that monoclonal antibodies are produced at an enhanced rate during the G_0 phase of the cell cycle (12–14). A model for antibody production based on this cell cycle dependence and traditional Monod kinetics for cell growth has been proposed (15). However, it is not clear if this cell cycle dependence carries over to recombinant CHO cells. In fact it has been reported that dihydrofolate reductase, the gene for which is coamplified with the gene for the recombinant protein in CHO cells, synthesis is associated with the S phase of the cell cycle (16). Hence it is possible that the product formation kinetics in recombinant CHO cells is different from that of hybridomas.

3. Cell Culture Processes

A wide variety of mammalian cells are used in industrial practice. The scale of operation and product characteristics also vary considerably. To accommodate this diversity in cell lines, scale and products, several cell culture processes have evolved. Commonly used processes include batch (or fed batch) suspension culture, continuous perfusion culture, and microcarrier systems as well as a few other systems developed to meet specific needs. Figure 1 schematically illustrates the configuration of a few of these culture systems. Table 4 summarizes the pros, cons, and some typical applications of these technologies.

3.1. Batch Suspension Culture. The batch suspension culture is perhaps the simplest technology available. It is adapted from traditional bacterial fermentation (qv) technology by changing the impellers to low shear marine propeller type, thus reducing the shear forces to which cells are subjected. Oxygenation is achieved either by direct sparging, if the cells are not subject to damage by bursting bubbles or are protected by surface-active agents such as pluronic polyols, or by membrane oxygenation where gas-permeable tubing is inserted into the fermentor.

Most hybridomas can be grown in batch suspension culture. Recombinant CHO cells can also be adapted for growth in suspension. However, CHO cells often require serum or expensive serum proteins to grow in this manner. The

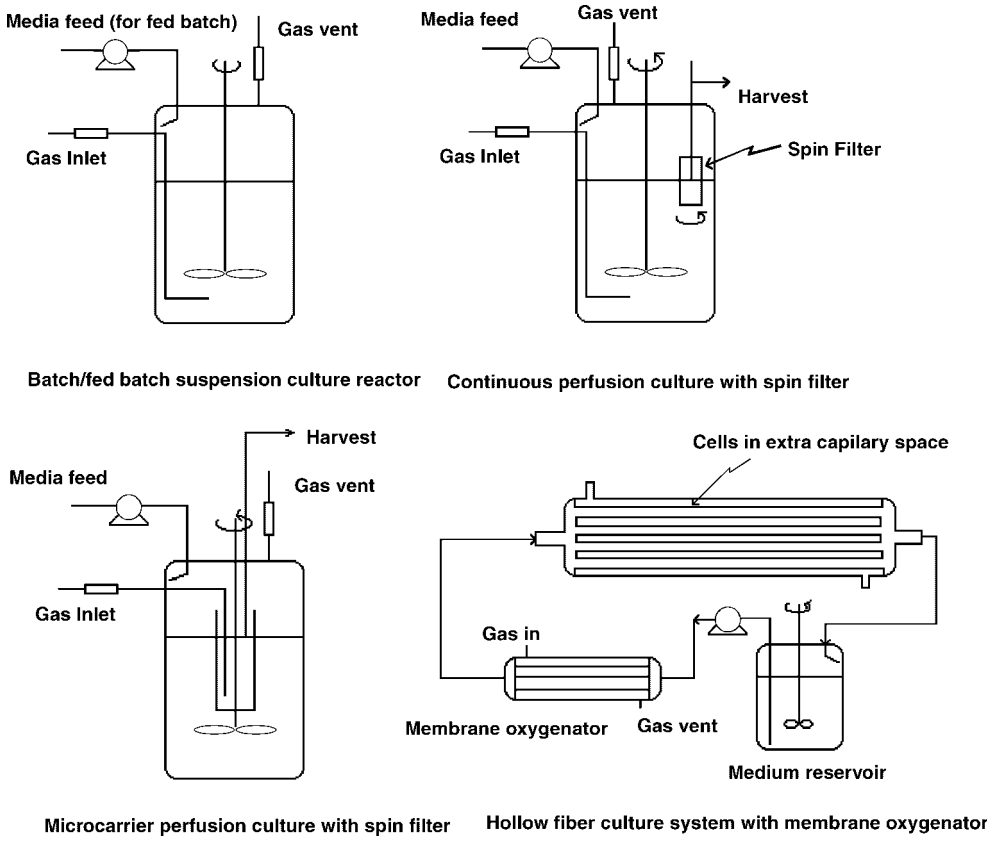

Fig. 1. Commonly used cell culture processes.

applicability of this process hinges on whether an inexpensive serum-free medium can be developed for the cells in question and whether the proteins used for serum-free media development, eg, bovine serum albumin [9048-46-8] can be effectively separated from the product during downstream processing. Viral vaccines are often produced in suspension culture reactors because the product is isolated from the cells and presence of serum is not a hindrance to purification. For example, FMD vaccine is produced in 3000-L batch suspension culture reactors (17). Genentech produces tPA in 12,000-L batch fermenters using recombinant CHO cells. Batch suspension culture is also used for large-scale production of monoclonal antibodies. Celltech has scaled up airlift suspension culture reactors to 2000-L scale for the production of monoclonals (18).

A batch suspension culture reactor consists of a stirred tank typically having a height to diameter ratio of 1:1–3:1, fitted with a low shear impeller. The tank has a hemispherical bottom to avoid stagnant zones, because agitation level is very low, and the agitator shaft is either magnetically coupled to the motor or is coupled with a double mechanical seal to protect the culture from contaminating microorganisms. The vessel is pressure rated for in-place steam sterilization. Medium is filtered into the fermentor through a 0.1-μm absolute filter.

Table 4. **Commonly Used Cell Culture Technologies**

Technology	Pros	Cons	Applications
batch/fed batch suspension culture	simple, scaleable, homogeneous environment, high cell densities and product titers achievable with fed batch	not applicable to attachment dependent cells, *not* suitable for products sensitive to proteases and glycosidases	monoclonal antibodies from hybridomas and transfected myelomas, CHO cell based products, some viral vaccines, insect cells
continuous perfusion culture	useful when waste products are toxic to the cells or when product is sensitive to proteases or glycosidases, smaller reactor size, less expensive media	higher risk of lot failure due to contamination, equipment problems or clogging of filters, *not* always suitable for attachment dependent cells	monoclonal antibodies with hybridomas or transfected myelomas, some recombinant CHO cell products
microcarrier systems	suitable for attachment dependent cells, scaleable, use of traditional stirred tank equipment, low cost media	microcarriers are expensive, shear damage to cells, scaleup train can be cumbersome, clogging of screens can be a problem in continuous systems	large scale viral vaccine production from primary cells, some CHO and BHK cell based products
automated roller bottle systems	suitable for attachment dependent cells, fast implementation for low volume products, high reliability	scale-up is limited, higher cost of production	low volume/high value products including viral vaccines and recombinant CHO cell products
hollow fiber systems	low shear environment, low medium cost, smaller reactor size	scale-up is limited, nonhomogeneous environment, higher risk of lot failure	low volume monoclonal antibodies

Inoculum is added via a steam sterilized connection from the inoculum fermentor. The seed fermentor is typically one fifth to one-tenth the size of the production fermentor. Hence, for large-scale production purposes, a long inoculum train is required. Oxygenation may be effected by either direct sparging into the fementor or by using a coil of gas permeable tubing, either silicone or microporous Teflon tubing. The vessel is fitted with temperature, dissolved oxygen, and pH probes. The pH is controlled by addition of carbon dioxide (qv) to lower the pH and a suitable base, eg, dilute NaOH or $NaHCO_3$, to raise it. Dissolved oxygen is controlled by addition of air and/or pure oxygen. Insulated jackets are used for controlling the temperature. The vessel is manufactured from 316-L stainless steel and polished to a high degree, typically 240 grit followed by electropolishing, for ease of cleaning. Other materials used for seals, etc, are restricted to

medical-grade silicone, Teflon, Viton, and borosilicate glass to ensure that toxic materials do not leach into the culture and affect the cells.

The top left schematic in Figure 1 shows a typical cell culture process using batch (or fed batch) suspension culture. The downstream processing of harvest from the fermentor usually consists of a clarification step, either a centrifuge or a microfilter, followed by a concentration/diafiltration step using a tangential flow ultrafiltration membrane of an appropriate molecular weight cutoff. The concentrated protein is further purified by a series of chromatography (qv) steps. The downstream processing steps are usually similar regardless of the cell culture technology being used.

Batch suspension culture has many inherent advantages. It is relatively simple to operate and scale up and less susceptible to microbial contamination. The homogeneous nature of the process makes process control (qv) and optimization easier, and from a regulatory point of view, it is the easiest to define and validate. This is therefore the process of choice if the cells can be grown in suspension in a relatively inexpensive medium. A fed batch suspension culture process is a variation of this wherein concentrated medium is fed to the bioreactor over several days in order to sustain further cell growth and production. This enables the process to reach very high cell dinsities and product titers. A recent paper reviews a generic fed batch process that has been successfully applied to many different antibody producing cell lines (19). A disadvantage of the process is that labile products may degrade during the long batch periods, especially because of the presence of proteases released by lysed cells toward the end of the process. For such products, a continuous process having low reactor residence times may be more suitable.

3.2. Batch Microcarrier Process. A variation of suspension culture reactors is a system where the cell concentration is increased by perfusing medium through the reactor continuously while retaining the cells in the reactor by means of a spin filter device. This is shown schematically in the top right of Figure 1. Many other types of cell retention devices have been used as well. For example, an acoustic filter was used for perfusion cell culture of CHO cells at 100/L scale (20). A review of commonly used cell retention devices is available (21). A small fraction of cells are either continuously or periodically purged from the reactor to maintain high viability of cells. This type of system provides the benefits of high cell density and maintains a homogeneous environment for the cells. Since the medium is continuously perfused through the reactor, the product does not stay in contact with the cells for a long time. Therefore, this system is more suitable for products that are susceptible to degradation due to proteases and glycosidases. These systems can also be effectively used when there is a need to increase the capacity of existing stirred tank fermentors. Similar to batch suspension cultures, the applicability of the system is limited to attachment independent cells. The spin filter devices are also prone to clogging after several weeks of operation. External cell retention devices make the system more complex to set up and operate.

Applications of perfusion culture include production of factor VIII, and monoclonal antibodies by hybridomas (Bayer and Johnson & Johnson respectively). Production of monoclonal antibodies using a perfusion culture system with real time glucose control has been described (22).

For attachment dependent cells, the batch microcarrier process is the equivalent of the batch suspension process except for the fact that cells are attached to microcarriers. Cells attached on the surface of microcarriers are far more sensitive to shear forces than suspension cells because microcarriers are much larger than suspended cells. The damage to cells is theorized to be caused primarily by turbulent eddies when the eddy size becomes smaller than the particle size of microcarriers. Another mechanism for cell damage, especially at high bead concentration, is the bead to bead collision frequency. These mechanisms of cell damage in microcarrier cultures are discussed in the literature and have been quantified (23,24). Because of this limitation on agitation power input, agitator design is of great importance in microcarrier reactors. High efficiency impellers, which maximize flow and minimize shear, are utilized. The shear effects mentioned here are applicable to solid microcarriers where the cells are attached to the surface. Most recently, some macroporous carriers have been introduced that prevent the shear damage by providing attachment surfaces on the internal pores of the carriers. However, the attachment rate on such microcarriers is slower than that on solid microcarriers. This makes the porous microcarriers more suitable for long-term perfusion cultures.

Many microcarriers are available commercially for mammalian cell culture. The choice of microcarrier depends to some extent on the cell line being used and whether a batch or continuous process is being contemplated. Table 5 lists some of the microcarriers commercially available. The Cytodex family of beads is probably the most widely used. Cytodex 2 is recommended for cells having fibroblast-like morphology; Cytodex 3 is recommended for cells having an epithelial-like morphology. In long-term serum-free cultures, Cytodex 2 tends to retain cells longer than Cytodex 3, whereas the latter is useful when available inoculum density is low. In some cases, productivity is affected by the surface characteristics. For example, some cells have higher productivity on negatively charged polystyrene, eg, Biosilon, than the positively charged dextran. In designing a microcarrier process, it is recommended that a quick screening experiment be conducted to assess the suitability of the microcarriers available. A more extensive review of various types of commercial and noncommercial microcarriers and their applications is available (25).

In addition to attachment dependent cells, a batch microcarrier process may also be used for other cells that can grow in the attached mode because it allows the use of totally protein-free media. Many cells can survive for long periods of time in completely protein-free medium if attached to a surface. However,

Table 5. **Microcarriers for Stirred Tank Reactors**

Trade name	Manufacturer	Diameter, μm	Characteristics
Cytodex 1	Pharmacia, Sweden	147–248	dextran, high positive charge
Cytodex 2	Pharmacia, Sweden	135–200	dextran, positive charge
Cytodex 3	Pharmacia, Sweden	141–211	collagen-coated dextran
Biosilon	Nunc, Denmark	160–300	polystyrene, negative charge
Cultispher	Hyclone, United States	170–270	gelatin, macroporous
Bioglas	Solo Hill, United States	90–150	glass-coated plastic
Bioplas	Solo Hill, United States	90–150	cross-linked polystyrene

microcarrier reactors face several practical difficulties. First, if the cells are attachment dependent, generating sufficient inoculum for large-scale reactors involves trypsinization of microcarriers from a smaller reactor and reattachment in the larger reactor. The exposure to trypsin [9002-07-7] has to be controlled carefully in order to minimize cell damage. Second, the switch to a protein-free medium entails draining the reactor and refilling with fresh medium, requiring settling of the beads for long periods of time, especially in large-scale reactors. During this time, the cells may be deprived of oxygen and other nutrients and may be affected adversely. Hence, batch microcarrier processes are suitable for vaccine manufacture where presence of serum may not be a hindrance. Finally, the low shear requirements make oxygenation much more difficult than in suspension culture. Gas sparging tends to carry all the microcarriers into the foam layer because the beads tend to adhere to the bubbles as they rise through the liquid. One approach to solving this problem is to aerate the liquid in a rotating or vibrating cage, separating the microcarriers from the bubbles. The movement of the cage prevents the cage from getting clogged, especially in a perfusion system, with cells and microcarriers. Alternatively, gas permeable tubing may be used. Many fermentor manufacturers now offer caged aeration and perfusion systems as an option for cell culture fermentors. These include Applikon (The Netherlands), New Brunswick Scientific (United States), Cellex Biosciences (United States), and B. Braun (Germany/United States). B. Braun and Bioengineering (Switzerland) also offer silicone membrane oxygenation as an option. A good overview of microcarrier culture technology and an in-depth discussion of design issues and applications is available (26).

3.3. Microcarrier Perfusion Systems. Microcarriers may also be used in a continuous perfusion mode. In the perfusion mode, the reactor is constantly fed with fresh, sterile medium and product is harvested from the reactor at an equal rate. Perfusion systems have the advantage that the same cells can be maintained in a productive mode for several days, thus reducing costly downtime and the cost of expensive growth medium. Once the cells attach to the carriers and become confluent, they can be maintained in a productive mode in protein-free medium for several days or, depending on the cell line, for several months. High density perfusion systems are also useful for labile products, because low residence time in the reactor minimizes the exposure of the product to degradative enzymes and conditions. Continuous systems allow steady-state operation, which makes process control and process optimization easier. Continuous processes suffer two principal drawbacks. First, the risk of contamination is higher because of the increased complexity of the process. Additionally, contamination is much more costly for a continuous process than a batch process because the magnitude of product and labor loss is much higher. Second, if the cell line is subject to genetic instability, the reactor may lose productivity over a long period because of slow overgrowth of nonproducing cells. Further, it is harder to define a batch for regulatory purposes when the product is being made by a slowly changing population of cells, making validation of the process that much more difficult.

The bottom left schematic in Figure 1 shows a typical microcarrier perfusion system utilizing a rotating screen to separate the microcarriers from the harvest liquid. Oxygenation is accomplished by sparging gas within this cage.

This eliminates the possibility of damage to the cells from gas bubbles. The medium and harvest tanks are usually sized to hold at least 3 days worth of medium. Peristaltic or steam sterilizable diaphragm metering pumps are commonly used for pumping the medium and harvest. Steam sterilizable connections are used to maintain sterility during medium feed and harvest operations. Although the screen is shown as mounted on the agitation shaft, it is possible to attach the screen to a separate shaft driven by a second motor. The latter system has the advantage that the rotational speed of the screen can be optimized for microcarrier rejection without affecting the mixing and shear characteristics of the agitator. Other methods for retaining microcarriers within the reactor include settling towers, based on gravity settling of microcarriers, and "self-cleaning" static screens (27). In long-term continuous operation, cells tend to clump up and detach from nonporous microcarriers. For this reason, the macroporous carriers may be more suitable for long-term perfusion cultures.

Process design for microcarrier processes involves determination of the surface area of carriers required to accomplish the production in a given time. This can be translated from small-scale T-flask culture experiments. There is a practical limit to the surface area per unit volume that can be accommodated in a reactor. For example, for carriers having an average diameter of 150 μm, 30,000 cm^2/L is a reasonable limit. Beyond this limit the collision severity becomes a factor in cell damage. The reactor volume is determined based on these numbers and provision is made for the necessary oxygen transfer depending on the specific oxygen transfer rate of the cells. The procedures used for designing and scaling up microcarrier reactors have been described (28).

3.4. Fluidized-Bed Systems. So far we have discussed systems that have been used at large scale in industrial setting. However, there are many other systems that have been used for commercial purposes. For example, erythropoietin is produced commercially with an automated roller bottle system (29). Cells are grown on the surface of roller bottles in a growth medium. Cells are then shifted to a serum-free production medium for harvesting. All operations are carried out aseptically in a clean room environment by an automated machine providing a high degree of reliability and consistency. Although roller bottles are often considered to be obsolete because of the labor and space intensive nature of the process, automation makes them a viable process for products where the volume requirements are not very high. The Technology Partnership (Cambridge, England) offers robotic systems for the production of cell culture-derived products using roller bottles and T-flasks. Automated roller bottle systems can be used when the volume requirements are relatively small. Automation of the various roller bottle handling steps and media and cell manipulation steps provides a high degree of reproducibility and reliability to the process. This technology is also being used commercially for viral vaccine production with primary cells.

Another commonly used technology for small volume production of proteins is the hollow fiber system (shown schematically in the bottom right of Fig. 1). A hollow-fiber device consists of a bundle of hollow fibers, usually made of hollow anisotropic plastic fibers that allow diffusion of molecules smaller than a specified molecular weight cut-off, potted at both ends of a plastic shell (see HOLLOW-FIBER MEMBRANES). The cells are immobilized in the extra capillary space (ecs) of

the hollow fibers and the medium recirculates through the lumen of the fibers entering and exiting via headers at either end of the reactor. The cells are held in a static mode and grow to high tissuelike density in the ecs. The medium access to the cells is via diffusion and Starling flow. Thus shear forces on the cells are minimal. This system is especially suitable for cells that are extremely sensitive to shear induced injury. Another advantage is that the fibers can be specified such that the product, often a high molecular weight protein, can be retained within the ecs, while medium components and metabolic by-products can diffuse through. This arrangement allows for *in situ* concentration of the product to very high levels. Similarly, serum usage can be minimized by entrapping the high molecular weight protein components of serum in the ecs and using protein-free medium for perfusion.

Scale-up of this system is limited by the size limitation on hollow-fiber manufacturing. This system is also more suitable for the diagnostic markets where quantities of protein required are relatively low. A disadvantage of the system is that nutrient gradients are set up in the ecs leading to a nonhomogeneous environment making process control difficult. This problem can be solved by pressure induced flow through the ecs (30). However, this increases the complexity of the system. More recently, production of tPA by recombinant BHK cells in a microfiltration hollow fiber bioreactor has been described (31). Hollow-fiber devices are available through Biovest International (Englewood Cliffs, New Jersey). Hollow fiber systems are being utilized by Cytogen and IDEC pharmaceuticals for small volume commercial production of monoclonal antibodies.

In the last 20 years, many different processes were developed that for one reason or another never found significant commercial applications. These include the fluidized-bed system (32) and a ceramic matrix bioreactor developed by Charles River Biotechnical Services (33). Other companies have attempted to scale-up existing T-flask processes linearly by increasing the available surface area in a compact space. An examples of such a system is the cell cube bioreactor (34). A disposable bioreactor using wave induced agitation has also been described (35). These systems are commonly used in the laboratory environment for research purposes—but have not been utilized for commercial production.

4. Economic Aspects

The 2003 market for cell culture-derived products is expected to exceeded $15 billion/year. The market is expected to continue growing substantially throughout the next decade. Cell culture products include erythropoietin and its second generation product ARANESP, 2003 expected sales of ~$7.4 billion, for the treatment of anemia associated with kidney dialysis and chemotherapy, Rituxan, 2003 expected sales of $2.5 billion, for treating non-Hodgkins lymphoma, Remicade, 2003 expected sales of $1.6 billion, for treating rheumatoid arthritis, and Enbrel, 2003 expected sales of $1.3 billion, for treating rheumatoid arthritis.

4.1. Process Economics. Relative economics of various cell culture processes depend heavily on the performance of the cell line in a system and on the cost of raw materials, particularly the medium. Models are usually devel-

oped for the various processes using productivity data obtained from small-scale experiments (see PILOT AND MICROPLANTS). Often, for high value products, the process which ensures the shortest time to market may be the process of choice because of other economic criteria. This is especially true for pharmaceuticals (qv). Reliability concerns also often outweigh economic considerations in choosing a process for a high value product.

Continuous processes have lower labor costs but have higher failure risk. Batch processes can be started back up in a shorter period of time than can a complex continuous process. Batch processes are easier to take through the regulatory process than are continuous processes. Thus batch processes are often chosen for mammalian cell culture systems, even though continuous processes can offer significant cost advantages. Cell culture costs constitute only a small (10–30%) fraction of the overall cost of making a product. A detailed discussion of the economics of biotech products is available (36).

5. Regulations and Standards

Most of the products derived from cell culture technologies are for therapeutic or diagnostic applications and manufacture is regulated by the federal government through the Food and Drug Administration (FDA). The FDA requires that all drugs be manufactured in compliance with current Good Manufacturing Practices (cGMPs). Guidelines for cGMPs are provided through the *Code of Federal Regulations* (CFR) Title 21. Essentially, cGMPs require that all process steps and products be defined in a quantitative manner by the manufacturer, ie, specifications for all important processes must be developed and methods for testing and validating those steps must be identified. The FDA has published a guidance document for submission of relevant chemistry, manufacturing, and controls information for therapeutic recombinant DNA derived products or monoclonal antibody products for *in vivo* use (37). Other relevant guidance documents are also available at the FDA website (*www.fda.gov*) (38,39).

The biotechnology (qv) industry has no formal standards for equipment manufacture and quality control as of this writing. The American Society of Mechanical Engineers (ASME) has an active committee to devleop standards for bioprocess equipment (Bioprocess Equipment Standards Committee—located in www.asme.org).

6. Safety Considerations

The fact that cell culture-derived products are often injected into humans as therapeutic agents makes it imperative that there be no component in the final product that can pose a potential health risk to the patient. Health risks can be introduced into a product from many sources including: the cells themselves; raw materials, such as serum, media components, etc; materials used in purification, eg, antibodies; and external contamination. For a therapeutic product such risk factors are identified at the outset and ways of reducing them to acceptable levels are designed into the process. Before a product is

released by the FDA the manufacturer has to demonstrate this risk reduction by rigorous validation of the process.

Some of the cells used in manufacturing are continuous or "immortal." Many of these have been shown to be tumorigenic in immunosuppressed animals. The cells also contain endogenous materials such as retroviruses and nucleic acids (qv), both of which can induce tumorigenesis, and immunogenic foreign proteins. Serum used in media can also introduce adventitious agents such as viruses and mycoplasma into the product. Other process chemicals, including cleaning agents, are low molecular-weight compounds that may be hazardous as well. Purification chemicals, such as monoclonals used for affinity purification, can be immunogenic to humans. Some of the potential health risks in mammalian cell culture processes and the methods used for risk reduction include:

cells	microfiltration
retroviruses	irradiation, sonication, heat, solvents, etc
nucleic acids	chromatography
cellular proteins	chromatography, ultrafiltration
bacterial contamination	microfiltration
process chemicals	diafiltration with appropriate buffers
serum proteins	affinity/ion-exchange chromatography

Most of these methods are commonly employed in the downstream processing of the desired cell culture technology product. Hence, most of the time it is only necessary to demonstrate that the designed process is reducing the putative risk factors to acceptable levels. Validation methods employed for risk reduction are discussed in the literature (40). In recent years, the risk of transmission of BSE via animal derived raw materials has led many companies to source their serum from "BSE-free" countries such as Australia and New Zealand. This risk has also prompted development of animal product free media for cell culture.

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SUBHASH B. KARKARE
Scientia Consulting