Chapter 10

Upstream Processing

Objectives

This chapter provides an overview of the key elements for operation of a biomanufacturing upstream processing production area. Upon completion of this chapter the student will be able to:

- distinguish between mammalian and microbial upstream processing
- describe the contribution of the areas of dispensing, media preparation, and fermentation/cell culture to the upstream process as a whole
- describe proper methods to avoid cross-contamination in the dispensary, such as the use of airflow booths and following cleaning protocol
- explain the in-process controls for assessing media components
- illustrate the major types of bioreactors and associated instruments used for upstream biomanufacturing
- evaluate situations in order to make appropriate aseptic decisions
- obtain and interpret various assay values, apply the data, and determine and execute appropriate responses for the upstream process
- define and describe methods of contamination control that are directly influenced by the upstream biomanufacturing personnel, such as aseptic techniques for open processing, closed processing, and helium testing that can increase the biomanufacturing success rate
- identify critical process parameters for cell growth and protein production and understand how they are controlled by the biomanufacturing operator
- explain automated and manual cell counting techniques and determine accurate cell count values from given laboratory data
- describe processing steps for primary recovery/harvest
- apply filtration and centrifugation theory and mechanics to appropriate fermentation and harvest steps

Terms

Active Pharmaceutical Ingredient (API): any substance or mixture of substances intended for use in the manufacture of a drug (medicinal) product; when used in the production of a drug it becomes an active ingredient in the drug product

Ampoule: a small sealed vial used to contain and preserve substances that must be protected from air and contaminants

Analytical method: laboratory procedure used to analyze a sample

Animal-derived materials: Animal Sourced/Animal-Derived Material is defined as a raw material that is created from or processed in part from animals. Materials of Special Consideration are not considered animal-derived; these include materials of animal origin defined as "Special considerations" as described in EMA/410/01. These are materials that are unlikely to be sources of TSE/BSE when produced in accordance with EMA/410/01 guidelines. Examples include collagen, gelatin, tallow derivatives, milk and milk derivatives, wool derivatives, amino acids, etc.

Batch: a specific quantity of material produced in a process or series of processes that is expected to be homogenous within specified limits; may also be referred to as a "lot."

Batch record: a record of all materials and proportions used to produce a batch

Bioreactor: a device or system meant to grow cells in cell culture

Cell Bank (working): cells grown from those maintained in a master cell bank with well-characterized stability and uniformity

Centrifugation: the process of separating the lighter constituents of a solution, mixture, or suspension from the heavier constituents by centrifugal force

Clarification: the removal of small amounts of fine, particulate solids from liquids

Clean In Place (CIP): a method of cleaning the interior surfaces of pipes, vessels, process equipment, and associated fittings without disassembly

Conductivity: a measure of a material's ability to conduct an electric current

Cryovial: a small vial designed for the storage of biological materials, cells, etc., under extremely low temperatures

Culture initiation: at the beginning of each batch of a campaign, a vial of cells is transported to an inoculum prep room; the culture is then initiated by thawing the vial.

Dewar: a storage vessel which provides thermal insulation by interposing a partial vacuum between the inside and the outside walls of the vessel; cell cryovials are stored in liquid nitrogen within a Dewar

Dissolved Oxygen (DO): the amount of oxygen gas that is dissolved in the media and is available to cells

Feed stream: consists of cells and culture media; the feed stream is cooled and microfiltered at a temp of 4 degrees Celsius

Fermentation: biopharmaceutical fermentation involves the culture of yeast or microbial cells for the purposes of protein drug production; most fermentations are aerobic; this is not to be confused with biochemical fermentation, which is the breakdown of complex carbon compounds into simpler compounds of water, carbon dioxide, etc.

Generation number: the population-doubling level of the cell bank

HEPA: a commonly used acronym for High Efficiency Particulate Arrestance

HVAC: a commonly used acronym for Heating, Ventilation, and Air Conditioning used for indoor environmental control

Lot: a specific quantity of material produced in a process or series of processes that is expected to be homogenous within specified limits; may also be referred to as a "batch."

Non-Animal-Derived Materials: materials used in the manufacture of biopharmaceuticals that are accompanied by a Certificate of Origin (COO) stating that the material was not derived from animal sources

Permeate: product (including media and other proteins similar in size) smaller than the filter pore size that passes through the pores of the filter and enters the filter housing where it can be collected

pH: a measure of the acidity or alkalinity of a solution

Retentate: consists of rejected species (cell debris, proteins larger than the filter's pore size, etc.) that remain in the recirculation loop until removed

Spinner flask: a flask used to culture cells; the side arms of the flask are used for drawing samples from the culture and transferring the culture from one vessel to another

Steam In Place (SIP): SIP occurs when bioreactor vessels and piping are sterilized with clean steam to establish a sterile boundary

Upstream Processing: process incorporating dispensing, media preparation, and cell culture

WC: inches water column; a measurement of pressure

Introduction to Upstream Processing

Overview

The upstream biomanufacturing technician's tasks center around microscopic, living cells. The main objective of upstream manufacturing is to create the environment necessary for cells to make the target protein. These proteins serve various medicinal purposes. Biomanufacturing is used to produce products such as therapeutic proteins, antibiotics, hormones, enzymes, amino acids, blood substitutes, and alcohol. They can be used as antigens in vaccine production or function as therapeutic hormones, enzymes, or antibodies against a particular disease or disorder. Lymphoma, rheumatoid arthritis, diabetes mellitus, and growth hormone insufficiency are a few of the human afflictions that biopharmaceuticals can successfully treat by reacting with specific protein targets. The product of interest may be excreted from the cells (generally in the case of mammalian cells) or may be intracellular (generally in the case of bacterial cells). In biomanufacturing, **upstream processing** is the growth of either bacterial or cell culture-based protein products, referred to as **microbial fermentation** or mammalian cell culture respectively.

These disease-fighting proteins are collectively called **Active Pharmaceutical Ingredients**, or **APIs**. The efforts of upstream technicians may be focused on one of the stages leading up to large-scale production: discovery research or clinical trials. Or the technician may be helping to produce a substance that has passed all phases of clinical trials and is approved for commercial sale. In clinical or commercial production, exacting attention to every processing step is critical to a successful batch. The **batch record** is the set of instructions that must be followed exactly as written and documented immediately following each action.

The remainder of this chapter will outline how the cells are obtained and grown to produce the protein of interest and will discuss the subsequent initial separation of the desired protein from the spent cells.

Cells and proteins

The cells that upstream technicians will care for in every step of the upstream process have been intensively studied for many years. The Dewar room, where cells are kept frozen in liquid nitrogen (LN_2), may be the most valuable room in the biomanufacturing plant. A **Dewar** is a specialized vessel that provides the environment to keep cell processes in temporary frozen suspension. The cell lines, contained in cryovials in the Dewar vessels, have been subject to many years of exacting research. Since they have been intensely studied, they are of great value. Why are these cells unique? First, the cells must have the required "transfected" gene that expresses the desired API. Transfection of a cell introduces a set of genetic instructions that are not native to the cell that codes for the desired protein. This gene is usually linked with a gene that imparts special survival abilities to the cells that possess it. Thus only those cells with the transfected gene will survive.

The growth medium, which will be discussed later in this chapter, provides all the nutrition cells need within a narrow window of environmental conditions for optimal expression of the target protein. This "selective pressure" can be produced by several approaches, including either the addition of a particular chemical agent added to the medium that kills cells without the gene or

the presence of a specific gene that allows cells to survive in media lacking a required nutrient. In either case, cells with the transfected gene will be able to thrive and make the target protein, whereas cells without these special genetic abilities will die out. Thus upstream efforts are focused on cells that promise maximum yield of the protein product. Proteins expressed by these cells are called recombinant proteins. Recombinant proteins can be either extremely small or quite large, as in the case of a monoclonal antibody. The average molecular weight (MW) of a monoclonal antibody is on the order of ~100,000 Daltons (100 kD).

Whether a technician works with small, rapidly dividing bacteria called “microbial culture” or with slower dividing mammalian cultured strains, the steps toward creating the environment necessary for optimal protein expression are similar. Microbial cells are prokaryotic cells and are much simpler in construction. These cells lack internal membranes and thus do not have the cellular machinery to secrete the desired API protein into the medium. Hence, the protein remains inside the cell. The cell must be lysed (split open) during harvest.

Mammalian cells are eukaryotic in origin; they contain a nuclear envelope and inner membrane system with the cellular machinery to secrete the API into the growth medium. APIs from mammalian cells can be larger and more complex since eukaryotic cells are capable of substantial post-translational modifications of certain proteins. Upstream technicians work with mammalian strains such as Chinese Hamster Ovary cells (CHO) and Non Secreting Null (NS0) mouse myeloma cells (NSO). Rodent cells are used because they have the unique ability to divide continuously in culture. The CHO cell line is the most common mammalian cell line successfully developed for the production of therapeutic proteins. Monoclonal Antibody (Mab) proteins are therapeutic proteins that have the qualities of natural proteins, thus there is a low probability for a patient's body to reject the drug.

From a manufacturing perspective, mammalian cell culture requires careful control of many variables to ensure successful production of the protein. These variables are outlined in the following sections. Microbial culture differs from mammalian culture in that there is less risk of contamination and, bacteria, with simpler cellular machinery, divide much more quickly. The time it takes for one microbe to divide is often less than 30 minutes, where mammalian cells require upwards of a day to double. Thus the time bacterial cells spend in culture is less.

Cells are grown in a media suspension within tanks or bags called bioreactors (mammalian) or fermenters (microbial). A unique aspect of these cells is that they can be grown in suspension within bioreactors rather than in adhered sheets. Most cells demonstrate a preference to grow in contact with other cells but some cultured cells can be selected to lose what is called “adherence dependency” (sometimes called contact inhibition) and grow in suspension. This flexibility allows for optimal growth of cells in culture to high densities in a large-scale bioreactor. Bioreactors and fermenters can vary greatly in size and can hold from 3 liters (3L) to 20,000 liters (20,000L) of growth media.

Upstream Processing Areas, Equipment, and Systems

Dispensing room

Materials needed by the biomanufacturing process to make product are weighed and measured in a traceable, controlled, and sanitary area prior to their use. Optimally, raw materials are received into the manufacturing facility in packaging which is suitable for cGMP processing areas (e.g., glass or plastic with no cardboard or other fiber shedding materials). In cases where larger containers of bulk raw material are received, the exact amount required for the production batch will need to be removed from the bulk, packaged appropriately, and then transferred to the point of use. The dispensing room is the location where raw materials for use in the production process are weighed or measured.

It is critical to mitigate risk of cross-contamination of one material to another during the dispensing process. Per regulatory guidelines, **non-animal-derived** and **animal-derived** raw materials must be segregated to reduce risk of exposure to adventitious viruses. To achieve this, dispensing booths are often dedicated to either animal-derived material or non-animal-derived material. For an extra level of precaution these materials can be weighed in separate rooms.
In some biomanufacturing facilities, the dispensing process can be electronic. One operator can perform the weighing using a scale that provides electronic verification and recording of the data. However, in many companies a manual system is still used. An operator and a verifier perform the weighing then record the data on a paper document. Verification is performed so that a second qualified individual can ensure that the required amount of the correct material is dispensed. Depending on the size of the manufacturing facility, raw materials dispensed can range from extremely minute to quite large quantities. For example, 1 milliter (1 mL) of a liquid supplement or 400 kg of a salt for a buffer may be dispensed. Personnel are trained to use equipment such as a drum inverter post hoist or a vacuum tube lifter for large solids handling.

There are certain performance characteristics required of the dispensary room and dispensary booths (also called containment booths). The air cleanliness classification of the dispensing room is Class 100,000 (EMA Grade C, the European classification) which is achieved using **HEPA** filtration of the air supply to the room. The **HVAC** system also maintains the room at a positive pressure with respect to the surrounding rooms and corridors (generally +0.10 inches water **column (wc), positive pressure**). The positive pressure keeps the airflow in the room blowing outward into adjacent rooms and airlocks when doors are opened. The room pressures are continuously monitored by the building automation system so that data is always available to demonstrate that the dispensary room is meeting the requirements needed to maintain a controlled state. Additionally, the pressures are tied to alarms so that personnel will be notified when the room is not meeting requirements. There should also be procedural control to stop dispensing in the booths in the event of such an alarm. Cleaning of the area and Environmental Monitoring should be performed once the alarms for the area are corrected and the room is operating in a stable manner. Additionally, several layers of gowning are generally required in a Class 100,000 room and takes place in an adjacent personnel airlock.

The dispensing room has airflow booths in which bulk raw material is opened to weigh/measure into required amounts. The dispensing booths circulate 90 percent of the

airflow through a pre-filter and HEPA filter bank. The remaining 10% of the booth's total airflow is returned to the room after HEPA filtration through a perforated grill at the top of the booth and all particulates are captured prior to any air return. The same amount of air is drawn into the booth from the room and is considered Grade C clean air from the main dispensary room.

Each booth should have separate HEPA filters to prevent cross-contamination during raw material dispensing. The booths are for containment and are certified to contain airborne particulate generated within the booth to a demarcation line at the front of the booth. All instrumentation and HEPA filters should be re-calibrated and re-certified on a periodic basis, generally every six months.

A best practice to prevent cross-contamination is to have only one raw material in the booth at a time. Cleaning should be performed based on the type of material dispensed and the assessment of risk for cross-contamination. Gowning required during dispensing generally includes a face mask, sleeve covers, and an additional gown suit prior to opening any materials. The dispenser dons a second layer of gloves prior to dispensing material, with the outer layer of gloves changed between each lot of material being dispensed.

All weighing utensils, such as scoops, pipette tips, spatulas, weighing boats, and graduated cylinders that are opened for dispensing of the material are discarded or properly cleaned after each raw material is dispensed. If spillage or excess material is seen on the exterior of the original vendor container, the exterior of the container is wiped down prior to removal from the booth or hood.

CIP/SIP systems

Vessels used during the biomanufacturing process and all associated piping/hoses must be free of any foreign substances prior to use. Foreign substances include cell debris, medium, cleaning chemicals, and even the target protein from a prior batch. As most bioreactors are multi-use (and may be multi-product), any substances inadvertently left behind can contaminate the next batch. Product left behind from the previous run could encourage microbial growth. Since APIs are drugs intended for introduction into patients, it is critical that the manufactured product be pure. **Clean in Place (CIP)** and **Steam in Place (SIP)** are validated cleaning and sterilization procedures that ensure the bioreactor is safe for use.

CIP involves automatic cleaning of processing equipment, vessels, piping, and in-line devices with minimal manual setup and shutdown and little or no operator intervention during cleaning. Sprayballs are used to clean the inner surfaces of the tank during CIP. Sprayballs are located within a vessel and have precisely located holes that ensure the cleaning solutions contact the entire interior surface of the tank.

Chemicals used in CIP are usually strong base solutions (such as potassium hydroxide) that are applied over all surfaces using the spray balls, followed by rinsing and the application of a strong acid such as phosphoric acid. These substances are all rinsed away by a final spray of high purity grade water so that no substances remain. The CIP skid uses conductivity to measure the content of cleaning fluids to ensure proper cleaning. Rinsewater must meet the threshold for conductivity to be considered clean. The acceptance of the CIP is based on reaching required conductivity or resistivity set points (targeted values) for specific durations; the set points ensure that consistent bioburden and TOC reduction is achieved.

SIP occurs when bioreactor vessels and piping are sterilized with clean steam to establish a sterile boundary. The sterile boundary defines the areas that are kept sterile during cell culture operations and includes the bioreactor vessel and all process piping up to either a major isolation valve or a gas filter. Sterilization is critical to prevent contamination. After sterilization is complete, the system must remain pressurized to maintain sterility. If the system pulls a vacuum, the SIP is repeated.

Media preparation area

Technicians working in media preparation prepare media with precise recipes required for each bioreactor stage of scale-up from inoculum (thawed cells introduced to the media) to harvest, where cells are separated from the media and the target API. As a human body requires a certain amount of carbohydrates, fats (lipids), and proteins in a diet to remain healthy, cells must receive proper nutrition to produce the protein product. Major components of media include:

- a carbohydrate energy source such as glucose
- a nitrogen source such as amino acids
- lipids often in the form of the subunit fatty acid

Some cell lines require supplemental feeding of cholesterol, a major structural component of cell membranes, along with fatty acids. Cells also require trace minerals in the form of salts, just as a human tissues and organs require specific minerals.

Animal-derived serum has often been a component of media, providing many nutrients required by cells. There is a growing trend, however, toward animal-component-free media, or chemically-defined media. This reduces the threat of adventitious animal viruses that may be contained in animal-derived substances.

In most manufacturing plants, media components (usually in powder form) are collected by a dispensing team and sent to the media preparation area. As with all steps in the manufacture of biopharmaceuticals, it is critical that the correct components are used. Technicians must check each item through verification of the lot number to ensure that the correct material has been sent.

Media preparation is typically done in tanks, in which the powdered media is introduced to a high-purity grade of water for injection (WFI). Technicians follow a batch record with each step. Since tanks are used repeatedly they must be cleaned between uses and the technician must ensure that the tank's most recent cleaning was performed and is within expiry. CIP and SIP cleans, while very effective, are only given a certain window of time and must be repeated if any step occurs outside the required time parameters.

Prior to introducing the cells to culture, it is critical that the powder be dissolved and the media be homogeneous, and it is essential to allow time for proper mixing. Media must be visibly uniform in consistency throughout so that the cells' environment is optimal for growth. For each step of scale-up, the media contains all the nutrition and selective agents that the cells require for optimal expression of the target protein. This window is called the "target range," and the parameters measured include:

- pH (degree of acidity or alkalinity)
- conductivity (concentration of charged particles called ions)
- glucose
- osmolality (a measure of particle concentration)

pH

pH expresses the degree of activity of an acid (H^+) or base (OH^-) in terms of hydrogen ion activity. pH is defined as the inverse log of the hydronium ion activity (pH = -log [H_3O^+]) and is measured on a scale of 0–14. A pH of 7 is neutral; a pH less than 7 is an acid; and a pH greater than 7 is a base. Figure 10-1 illustrates a typical pH scale.

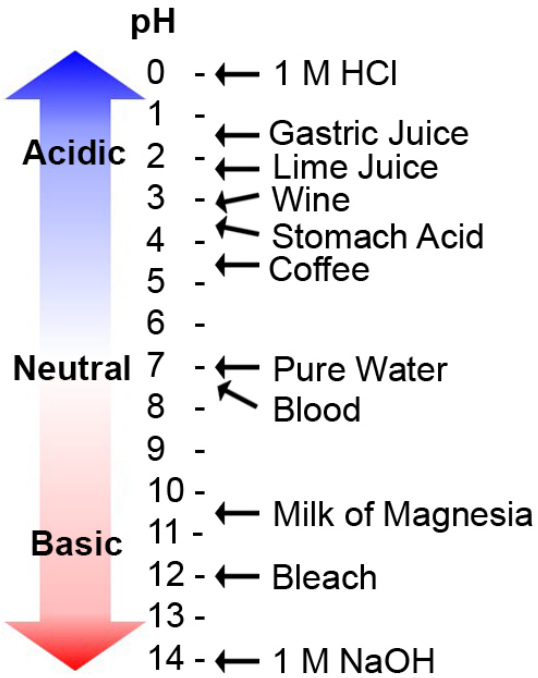


Figure 10-1. pH scale

Conductivity

Conductivity is a measure of the ions dissociated in the solutions. Conductivity measurements are important in CIP, as they reveal the purity of the rinse water. CIP cleaning uses acids and bases to clean tanks and is followed by rinsing with WFI. Conductivity measures the level of any residual acid or base ions left in the rinse water-the higher the concentration of ions in solution, the higher the conductivity. It is most often measured in microSiemens per centimeter (µS/cm).

Glucose in cell culture

Glucose is a water-soluble sugar added to all cell culture media. Cells derive their energy from glucose as a primary fuel. Energy derived from glucose is stored in the form of “high-energy” phosphoanhydride bonds in adenosine-5'-triphosphate (ATP**).** The amount of sugar ranges from 1 g/L (5.5 mM which is the normal blood sugar level *in vitro*) to 10 g/L (55 mM) and is monitored during processing. There are several methods for the determination of glucose available as an energy source.

Osmolality

Osmolality is defined as the number of osmoles of solute particle per kilogram of pure solvent and is represented in milliosmoles per kilogram (mOsm/kg). An osmole is the number of moles of a substance that contribute to a solution's osmotic pressure; the number of osmotically active particles exerting an osmotic pressure of one atmosphere in 22.4 L of solvent at zero degrees Celsius. An osmometer typically uses freezing point depression to measure osmolality.

Cell Culture/Fermentation

The goal of upstream biomanufacturing is to grow cells that will produce a desired protein, the protein being further purified during the downstream processing steps (see *Downstream Processing* chapter). The stages within the upstream biomanufacturing area are generally referred to as inoculum, bioreactor stage (seed and production), and primary recovery.

Environment necessary for cell growth

All living things grow by cellular division, and the same applies to fermentation or cell culture - the cells divide and continue to multiply. Just as humans need certain things to continue to live (food, water, O_2, a specific body temperature, etc.), cells in culture settings also require a specific environment to support growth. The upstream biomanufacturing facility is set up to achieve this. The growth medium is prepared and used to provide food; cell culture vessels are used to control gases and temperature for the cell growth. During growth the media, which are initially clear, will become opaque as the cell density increases over time. To determine how well the cells are growing in culture requires that the medium is sampled and the cells accurately counted. Each cell culture/protein product will have slightly different processing steps, but the general flow is outlined below.

Four distinct growth stages have been described for primary cells maintained in culture:

1. cells adapt to the *in vitro* environment, which is referred to as “lag phase”
2. cells undergo an exponential growth phase called the “log phase” or exponential phase
3. the relative rate of cell growth and death balance each other, leading to a growth plateau or peak
4. the rate of cell death exceeds the ongoing rate of cell proliferation and the growth curve declines, “death phase”

Throughout this cycle the cells process through a number of generations, with the doubling from the original seeding cell concentration constituting one generation. Depending on the organism, the growth cycle can vary broadly. For example, *E. coli* fermentations (doubling time: 20 minutes) are complete in 24 hours, whereas a mammalian cell culture takes 6–20 days (CHO cell doubling time: 14–17 hours). Figure 10-2 illustrates the phases of the growth cycle of a cell culture.

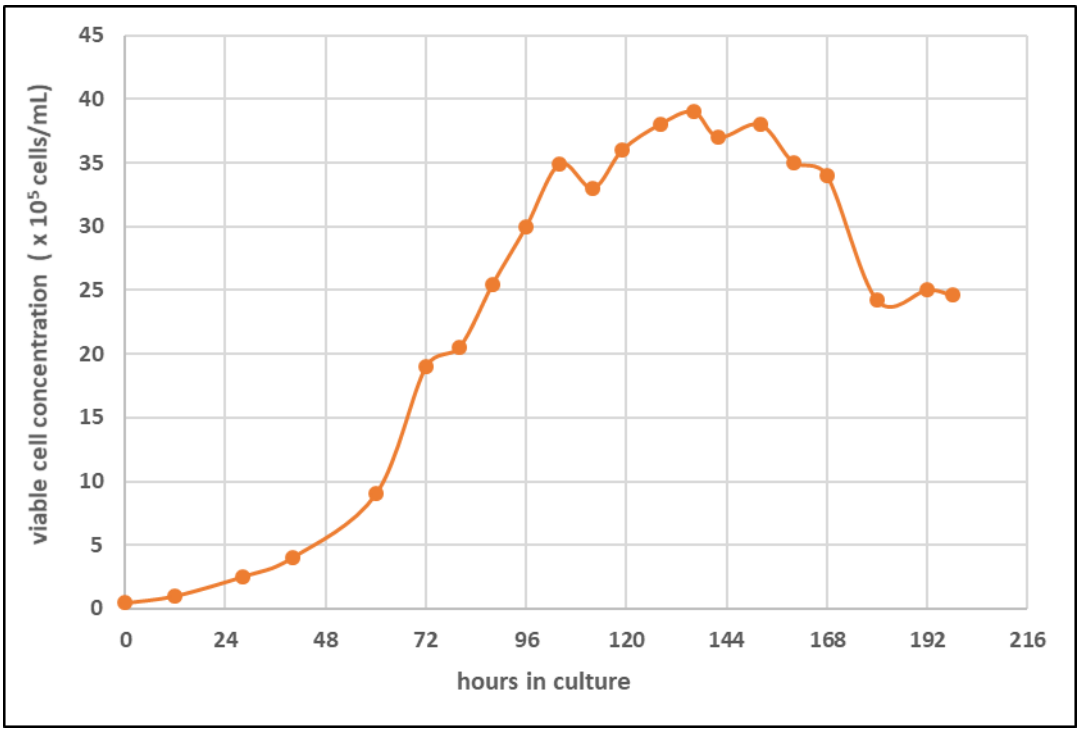


Figure 10-2. Distinct phases of the growth cycle of a production bioreactor cell culture

Cell banking

Cells can be stored for long periods of time at low temperatures. This permits cell stocks to be maintained for culture inoculum without having to resort to continual re-isolation of the needed cell from the primary tissue. The maintenance of a master cell stock also guards against loss of a cell line by contamination or genetic change that can occur by continuous subculture. It is often useful to store such cells at various passage numbers (**generation numbers**). This allows the monitoring of any genetic change that may have occurred during the culture period. Cells can be stored for long periods in the vapor phase of liquid nitrogen (-196 degrees Celsius) without deterioration. However, to prevent damage to the cell membrane by ice crystal formation, the cells are suspended in a cryo-protective agent (Figure 10-3). This normally consists of growth medium supplemented with 5% dimethylsulfoxide (DMSO) or 10% glycerol. DMSO is often preferred because of its greater rate of penetration into the cells; however, the exposure of cells to DMSO above freezing temperatures should be brief. For sensitive cell lines, fetal calf serum supplemented with DMSO (5%) can be used. Slow freezing and rapid thawing is recommended to maintain high cell viability.

Figure 10-3. Aseptic preparation of cell bank vials in a Biological Safety Cabinet (BSC)

A cell bank should have a full history of the cell line, as well as the criteria used to determine its authenticity (e.g., test results showing absence of bacterial, fungal, viral, and *Mycoplasma* contamination). Test results should also demonstrate cell line characterization, including isoenzyme analysis, chromosome analysis, and in some cases, DNA fingerprinting.

Inoculum

The inoculum stage involves the thawing of a frozen vial of cells (**ampoule**) and its introduction into a bioreactor. Placing cells in the bioreactor allows for scaling-up of the culture to meet cell density and volume requirements in order to inoculate the next stage in the process. The inoculum stage is comparable to the childhood stage in the production lifecycle.
The cells are cultured in specific conditions that promote the multiplication of those cells without actually producing the protein so that the volume is expanded to a level where protein production can begin. The cells are generally more vulnerable at this stage because in the majority of cases the inoculum stage involves open processing, which has more risk of contamination of the cells. To begin manufacturing a batch of API, one ampoule of frozen cells is generally released from the cell bank. These cells are thawed in a procedure referred to as Out Of Freeze (OOF) or Thaw. Once thawed the cells are added to prepared media and the inoculum phase of the process begins. Depending on the type of cells, this may take a few hours or a few weeks to reach the required volume and cell density volume expansion.

Cells can be grown in inoculum using a variety of equipment. Depending on the type of cell line, the culture may be monolayer (adhered to the surface of the vessel) or in suspension. If the scale-up will be done in a bioreactor, the cells will be in suspension.

When the culture reaches a cell density in which most of the media and oxygen have been exhausted, a new supply of growth medium and oxygen must be provided. The general practice for inoculum is to replenish these nutrients when the culture has reached 75–100% of its peak growth cycle. This is called a "subculture" or "passage." The growth of a culture will be adversely affected if cells are allowed to overgrow. Subculturing cells too early will result in a longer lag time before the next growth phase continues. The time between passaging cells depends on the growth rate and varies with the cell line.

If the cells have created a monolayer on the culture vessel, the first step in subculturing is to detach the cells from the surface of the primary culture vessel by trypsinization or by other mechanical means. The cell suspension is then reseeded into fresh cultures by adding new media to a smaller portion of the primary culture. This new culture becomes the secondary culture. The cycle repeats, and this culture may be subsequently subcultured to produce tertiary cultures, etc. Passaging of suspension cultures is somewhat less complicated than passaging of monolayer cultures. Because the cells are suspended in media rather than attached to a surface, it is not necessary to detach them before passaging.

A suspension culture is first grown in culture flasks such as a **spinner flask** (Figure 10-4) in a temperature-controlled incubator at approximately 35–38 degrees Celsius. The chamber can be humidified if required by the cell line. CO_2 gas can be applied in a passive manner, by CO_2 incubator, or actively via sparging the culture with CO_2 throughout the growth phase. Sparging involves bubbling a gas through a liquid. See the section ***Role of CO_2 in Bioreactors*** for more information. Cell bags may also be used to grow the culture, ranging from 50 mL to 200 L.

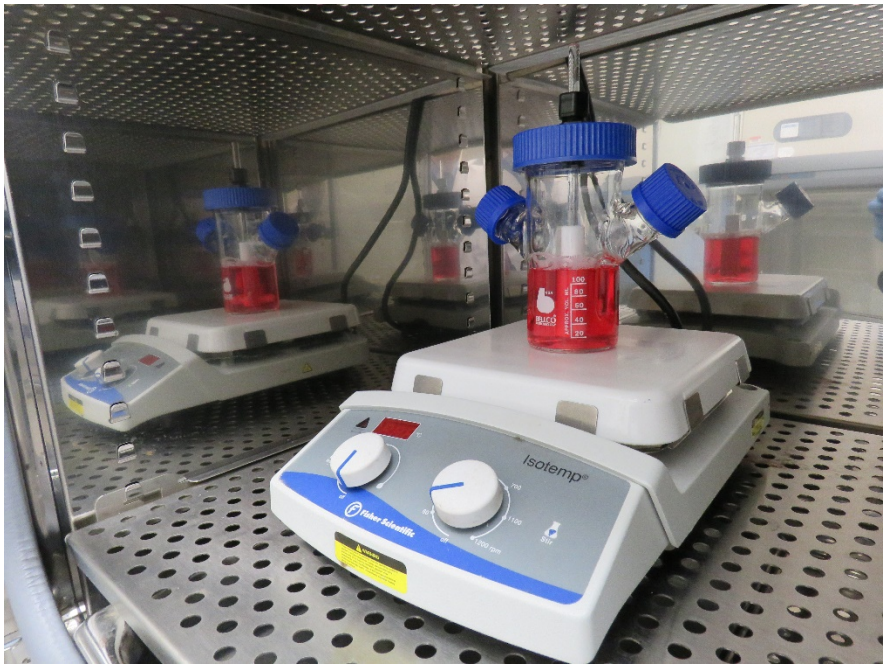


Figure 10-4. Spinner bottle containing cells and media on spinner plate within an incubator

Preventive measures must be taken to ensure that contaminating organisms do not get into the product. Any time a sample is taken from a bioreactor or whenever a container or vessel is opened, contamination is possible. During inoculum, aseptic technique is critical to prevent contamination of the culture. Preventive measures include ensuring proper cleaning of all materials, proper gowning (e.g., gloves, face masks, hair nets, etc.), and using proper aseptic techniques in the Biosafety Cabinet (BSC). BSCs are used during critical steps in fermentation. The BSC draws ambient air into the work area from the Class 100,000 room through HEPA-filters (Figure 10-5). When using a BCS one should ensure that the air intake grills are not obstructed, movements in the work area are slow and controlled, and unnecessary talking is avoided. One should also ensure that any vessel is only open for as long as necessary and one should never reach over an open vessel (Figures 10-6 and 10-7).

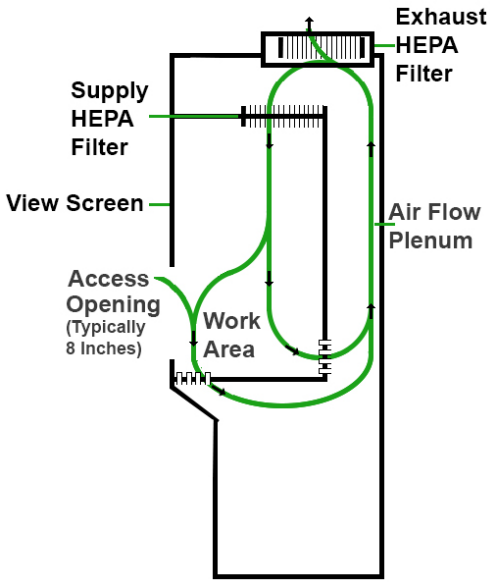


Figure 10-5. Airflow in a Biosafety Cabinet

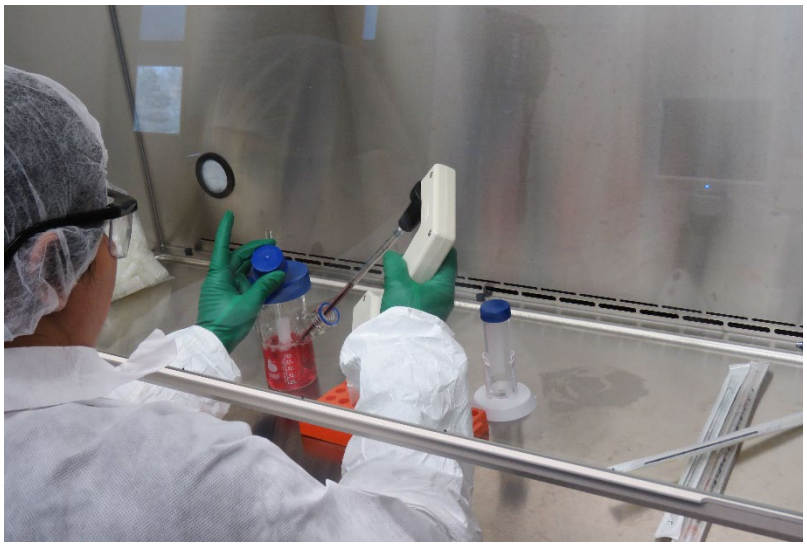


Figure 10-6. Open spinner flask in the Biosafety Cabinet

Figure 10-7. Proper opening of a pipette in a Biosafety Cabinet

Role of pH in cell culture

To achieve optimal cell culture conditions and maximize product yield, pH must be controlled during the processing. pH can affect growth and is referred to as a "critical process parameter." pH will not remain stable for a long period of time in an actively growing culture. The media generally contains bicarbonate, which when combined with the 5% CO_2 in the incubator air makes a buffering system to control the pH of the culture (Figure 10-8).

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

Figure 10-8.

In the bioreactor pH is measured by an in-line pH probe and controlled by an associated pH transmitter via the bioreactor controls system. Figure 10-9 depicts in-line dissolved oxygen and pH probes. The probes are inserted into the bioreactor and is connected to the processor via a cable.

In-line DO Probe

In-line pH Probe

Figure 10-9. In-line Dissolved Oxygen (DO) and pH probes.

If the pH is low, there is a high concentration of hydrogen ions, and if the pH is high, there is very low concentration of hydrogen ions. Along with the pH of the culture, pCO_2 is also an important factor to consider – pCO_2 is the partial pressure of CO_2 within the culture, and is a metabolic indicator. The cells use glucose and oxygen, converting them to carbon dioxide and water. Mammalian cells create CO_2 and lactic acid, which both lower pH. Bacteria and yeast utilize other similar pathways.

Alkaline solutions are used to increase the pH; the type of alkali used includes solutions of sodium bicarbonate, sodium carbonate, and sodium hydroxide. pH control is cell-density dependent and is generally not required at lower cell densities. Some processes may actually raise pH. Acids are used in the buffering to bring pH back down. Agitation, size and rate of air bubbles, sweeping and sparging, off-gassing, antifoam (used to control foam buildup), and other gases used in the culture growth all play a role in how CO_2 is controlled in the culture. pCO_2 can be lowered by sparging the bioreactor with O_2. Figure 10-10 depicts an air bubble in the culture and demonstrates how gas exchange occurs.

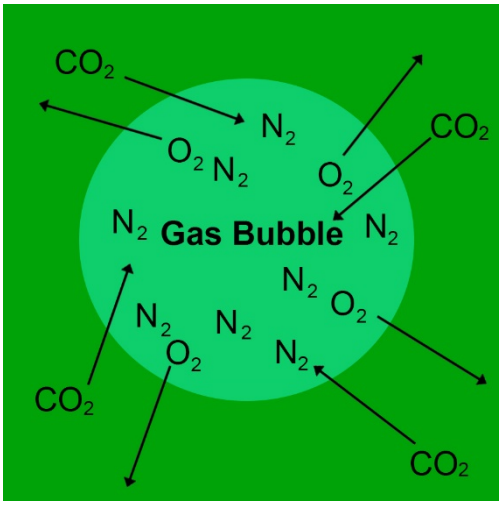


Figure 10-10. Gas exchange process

At lower volumes in the inoculum no sparging of gases generally occurs. CO_2 is supplied into the headspace above the culture liquid interface so that CO_2 in the culture will remain at equilibrium and be more effective as a buffering system to control pH. If CO_2 was not provided to the headspace, CO_2 could off-gas from the culture and impact the pH of the culture.

In bioreactors CO_2 is supplied through sparging within the culture so that CO_2 will then off-gas and build up in the headspace. Sweeping with air in the headspace overlay helps to remove the build-up of off-gassed CO_2 (Figure 10-11). Other gases are also provided through the sparger, including O_2 and air.

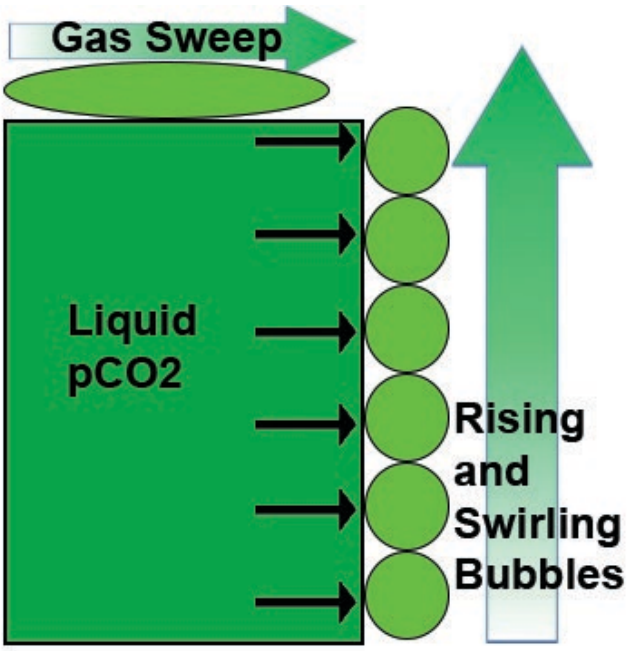


Figure 10-11. Sparging and gas sweeping

An impeller in the vessel will break up and disperse gas bubbles, thereby enhancing the mass transfer from liquid to gas. The rate of transfer will affect pH by influencing the level of CO_2 dissolved in the media. Agitation, provided by the impeller, can affect the size and shape of the bubbles, which can change how the gases transfer from bubble to liquid. Therefore, agitation can play a vital role in controlling the transfer rate of CO_2 in the culture. Two examples of agitators used are top-mounted and bottom-mounted.

- Top mounted: a motor that turns the agitator shaft and mixes the contents is mounted to the top of the vessel
  - This type of agitator relies on condensate or air to lubricate the mechanical seal between the shaft and the motor. Condensate is supplied to the seal through clean steam lines. Operation of the agitator relies on the blades of the shaft being submerged. If not, the shaft will wobble, causing severe damage to the agitator seal.
- Bottom mounted: a magnet drives the agitator
  - The drive coupling, being entirely magnetic eliminates not only the seals but also problems such as leakage, contamination, and maintenance. Such an agitator requires the liquid level in the tank to be above the agitator to prevent the overheating of contacting parts.

Foam in the culture can be created due to impellers, gas addition, cell growth, and strategies for controlling Dissolved Oxygen (DO). Foam can be detrimental to the culture, as it will limit the ability for off - gassing in the culture. This reduces the driving force of CO_2 transfer, preventing transfer of the CO_2 out of the culture. Too much CO_2 is bad for cell growth, as bursting bubbles kill cells. Antifoam is typically an oil-based substance that penetrates the micelle structures and allows the foam to stabilize. The antifoam interferes with this stability, breaking it down and causing the foam to dissipate.

Figure 10-12 depicts pH control in a mammalian cell culture. CO_2 gas is supplied to maintain the required pH setpoint in the early stages of the culture growth. As CO_2 gas usage decreases to zero, the demand of a base to maintain pH occurs since the culture growth creates CO_2; this needs to be neutralized in the culture to maintain the pH setpoint required.

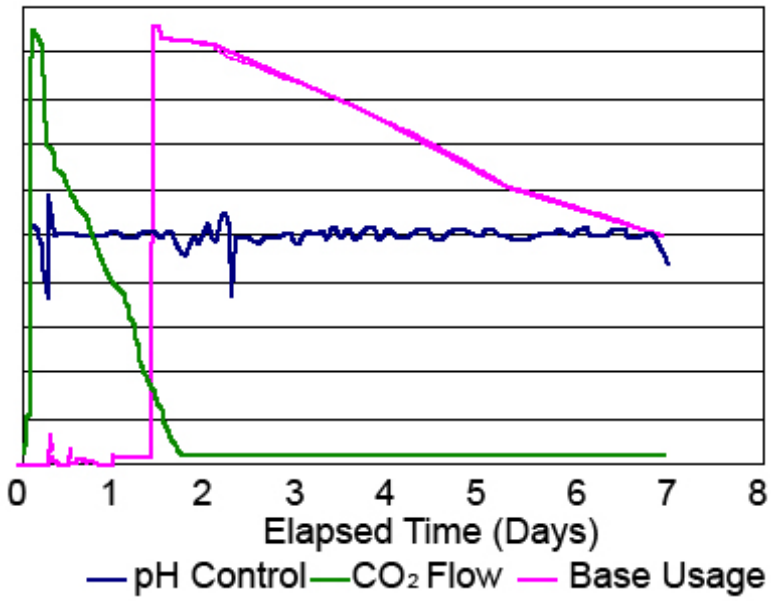


Figure 10-12. Example of pH control in a bioreactor

Bioreactors

The next stage in the production process for protein-based therapeutics is the use of seed and production bioreactors to create the conditions that optimize growth of the mammalian cells. Bioreactors are classified as either disposable or stainless steel. Regardless, all use the same basic principles in operation and use.

Disposable bioreactors

Disposable bioreactors are generally used in cell culture for mammalian processing. Introduced in 1998 in a format called the rocking bag (Figure 10-13 and Figure 10-14), disposable reactors are now also available in scales of thousands of liters using a stirred-tank disposable configuration.

Disposable bioreactors play a significant role in biomanufacturing operations and their use is increasing due to the development of high-titer processes, which allow for lower bioreactor volumes. With the advent of higher-titer processing, smaller volume batches require smaller bioreactors and less media.

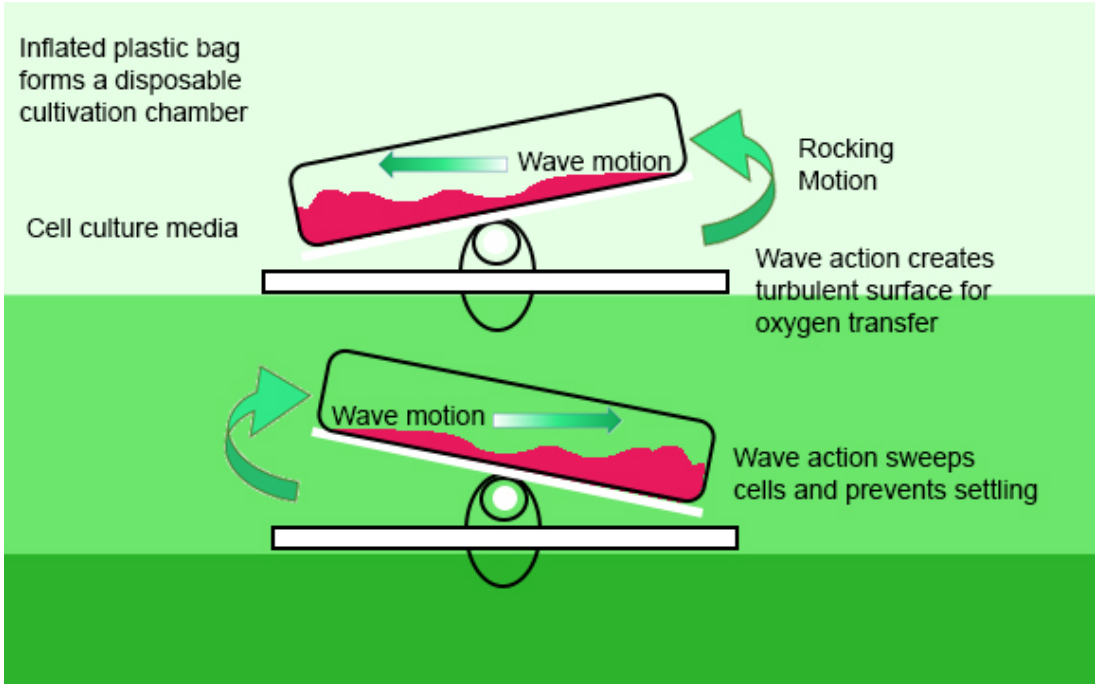


Figure 10-13. Wave bioreactor and its mechanics

Figure 10-14. Operator performing wave bag cell culture

Stainless steel bioreactors

This type of a bioreactor vessel is a double-walled, glycol-jacketed, sanitary pressure vessel designated to provide primary containment for the cell culture. There are four layers to the stainless steel bioreactor vessel:

1. Interior wall
    - provides the sterile product contact surface for the cell culture
    - made of stainless steel (316L) and designed according to sanitary principles
    - mechanically polished interior with a smooth sanitary finish
2. Jacket
    - a dimpled heat transfer surface welded to the outside of the interior wall
    - functions as a heat exchanger to control the temperature of the cell culture inside the bioreactor vessel
    - attached to an associated Temperature Control Module (TCM) linked to an automated control system
3. Insulation
    - located between the jacket and the outer sheathing
    - intended to reduce heat loss from the bioreactor vessel
4. Outer sheathing
    - visible outer layer of the bioreactor vessel
    - designed to enclose the insulation and glycol jacket
    - surface is typically rough and finished by mechanical grinding

Bioreactor mixing types

Types of bioreactors are also classified according to the type of mixing they perform. Mixing is a key component in the production process since it ensures a homogenous environment within the bioreactor by distributing nutrients evenly through the vessel and allowing control of critical parameters such as DO, pH, and temperature.

- Airlift: the mode of agitation is accomplished using nitrogen gas bubbling through a sparge tube within the bioreactor; to achieve proper mixing, these bioreactors contain a baffle that guides the nitrogen gas up through the bioreactor on one side of the baffle then over the baffle and down through the bioreactor on the other side (Figure 10-15).

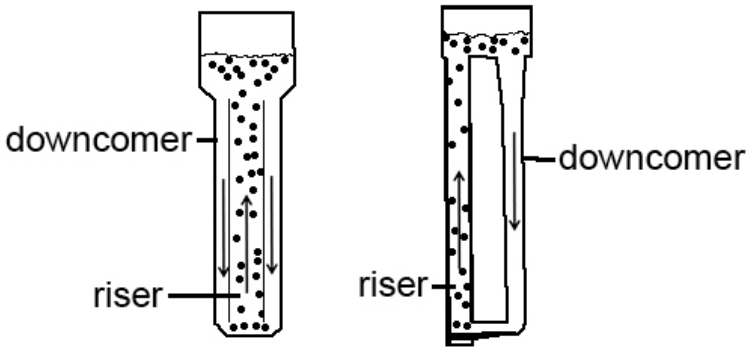


Figure 10-15. Air/nitrogen bubbles create a stirring effect on an airlift bioreactor.

- Stirred tank: an agitator impeller (similar to the propeller on a boat) moves the cells and media in the bioreactor; there can be one or more impellers on the agitator shaft.

Production strategy

The manufacturing process step involving bioreactors is further classified by the type of production strategy:

- Batch: the culture generally goes through the growth cycle of lag, exponential, plateau, and death; the production cycle is short in this type of strategy; protein is harvested one time at the end of the production cycle.
- Fed batch: the culture goes through the growth cycle of lag and exponential phases; additional feed (glucose and other nutrients) is added before the cells reach plateau phase, allowing the cells to continue growing and producing protein; eventually the cells will reach the plateau and death phase, completing the production cycle; protein is harvested one time at the end of the production cycle.

  Glucose and other carbohydrate energy sources are components of media. Figure 10-16 illustrates the relationship between glucose concentration and cell density. Cells use the energy source to grow and make the target protein. Measurements are taken of dissolved glucose. If no additional glucose is added, the amount of glucose and the cell density level will decrease with time.

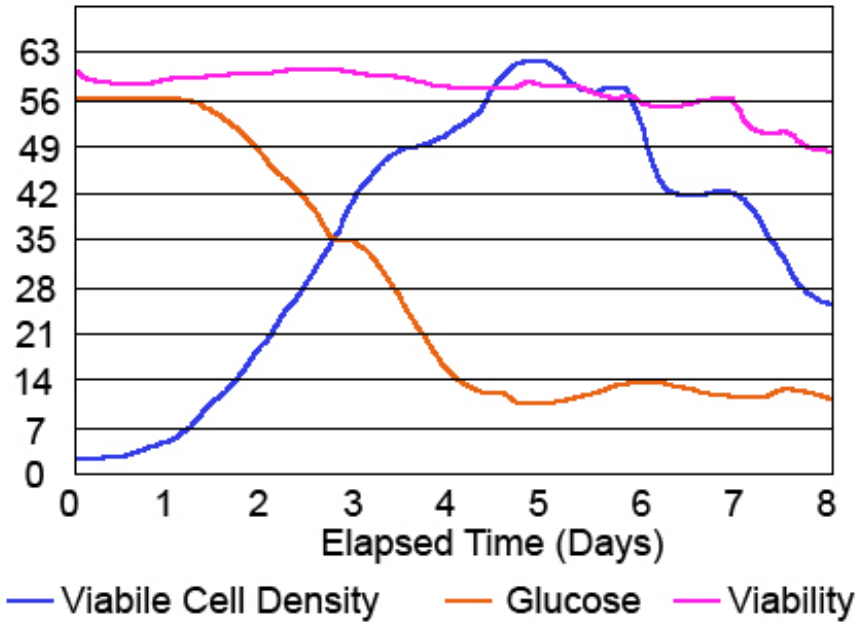


Figure 10-16. Glucose, a simple carbohydrate, is often the main food resource.

- Perfusion: the continuous growth of cells within a determined amount of time, with continuous harvest of product during cell growth achieved by a liquid-solids (cell) separation device within or adjacent to the production bioreactor; multiple harvests of protein are obtained from the bioreactor.

Bioreactor preparation

In the case of disposable bioreactors, a cell bag is sterilized (using Gamma irradiation) and set up to receive media and cells for cell culture. For stainless steel bioreactors, the bioreactor is prepared by cleaning and sterilizing using CIP and SIP. Leak detection is usually performed by pressure-testing the vessel with helium.

The biomanufacturing process employs several steps to ensure a closed processing system and eliminate the opportunity for cross-contamination or bioburden introduction into the product stream. For production steps where sterility is critical, a strategy is used whereby processing procedures and equipment minimize exposure of the product to the environment. The procedures/equipment used to support closed processing systems include SIP, sanitization, tube welding, tube sealing, and the use of autoclaved carboys/Gamma-irradiated bags for dropouts/bleeding in a sterile manner:

- SIP of Steam Through Connector (STC): used when adding supplements, antifoam, or feeds to bioreactor (Figure 10-17)

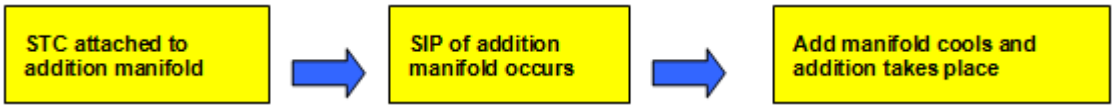


Figure 10-17. SIP of STC

Prior to an addition taking place, SIPs are used to ensure a sterile connection between the addition container to the bioreactor, harvest tank, hold tanks, etc. The SIP of the addition manifold is validated according to regulatory requirements. After attaching the STC to the additive manifold, a SIP is initiated, the goal of which is to meet certain criteria referred to as F sub O (the length of time at a predetermined temperature where it is impossible for living contaminants to survive). Both sterilization length and F sub O criteria may vary depending on the equipment involved. The SIP allows steam to enter the system and the temperature is monitored via temperature indicators placed throughout the system. Once SIP criteria are met, the steam supply is closed and the system is allowed to cool. After the cooling period the STC is welded to the addition container and transfer occurs from the addition container through the additive manifold into the vessel.

- **Tube welding**: tubing from one container to another are welded together to ensure a sterile connection (Figure 10-18).

Figure 10-18. Tube welding

When either additions are being made or samples are being removed from wave bags, bioreactors, harvest tanks, VI tanks, hold tanks, etc., tube welding is the general method used for connecting the addition/ sample container to the vessel. Tube welders are validated per cGMP industry standards. A Sterile Tube Fuser uses a non-reusable blade to cut, move, and join two pieces of thermoplastic tubing while maintaining sterile conditions in the interior of both tubing pieces. The blade will retract into the Sterile Tube Fuser unit, allowing the tubing to be placed in the tubing holders.

The two pieces of tubing are placed into the left and right tubing holders and clamped into position. The cover is then closed. A pushbutton (COVERLOCK) initiates the steps in the cutting and welding program. In the first step the blade is heated to 400 degrees Celsius and the temperature is maintained for 10 seconds to ensure that the blade is sterile. Next the blade is allowed to cool slightly. The heated blade is then moved so that it melts through the two tubing pieces simultaneously. Both pieces of tubing are forced against the blade to ensure that the tubing interiors remain sterile. Once the tubing has been cut, the machine slides the cut tubing pieces into alignment. The blade is then extracted while the tubing ends are pushed together. At all times during this extraction the tubing is in contact with either the other tubing piece or the hot blade. When the blade is fully extracted the weld is allowed to cool. The lid interlock is released when the weld is complete; the machine can then be opened. The connected tubing is removed, inspected, and ready for immediate use.

- **Tube sealing**: used to break a sterile connection while maintaining the sterility of the adjacent equipment (Figure 10-19).

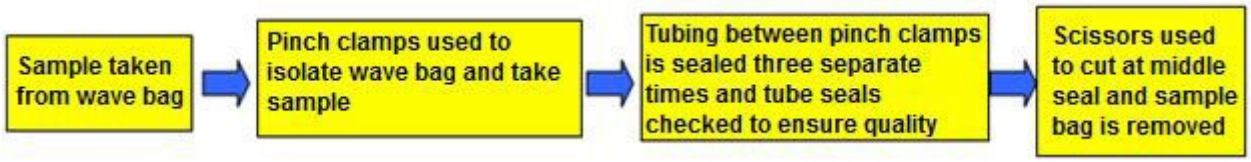


Figure 10-19. Tube sealing

After additions are performed or samples are pulled via tubing, tube sealing is the general method used for separating the associated containers/vessels. The tube sealer, validated per cGMP industry standards, is used to permanently seal tubing. This is done by clamping the tubing in a precise position between two heated jaws. The molten tubing is pressed together and cooled under high pressure, resulting in a permanent, leak-proof seal that is sterile. Almost any thermoplastic tubing, such as C-flex, PVC, or PharMed, can be sealed in this manner. The machine squeezes the tubing to be sealed to a precise thickness based on the material of the tubing and its diameter. The tubing is then melted by heat and cooled under pressure to form a seal.

Closed sterile connectors are used in the biomanufacturing process to allow two separate sterile pathways to be joined in a simple dry connection while maintaining the sterile integrity of both. The connector consists of a male and female connector, each covered by vented peel-away strip that protects the port and maintains the sterility of the sterile fluid pathway.

Monitoring and maintaining the culture

Once prepared, the bioreactor is filled with media, which is then equilibrated to meet setpoint ranges for temperature, DO, and pH. The cells are then inoculated into the bioreactor. During bioreactor processing, the culture is maintained by controlling temperature, DO, and pH target setpoints (pH was discussed earlier in this chapter):

Temperature: The commonly-accepted average human core body temperature is approximately 37.0 degrees Celsius (98.6 degrees Fahrenheit); the body has the remarkable capacity for regulating its core temperature somewhere between 98 and 100 degrees Fahrenheit. The cells in a bioreactor require a certain setpoint for temperature and control mechanisms to maintain the temperature within an associated acceptable operating range. Generally, a Temperature Control Module (TCM) on the bioreactor is used to achieve this.

Dissolved Oxygen (DO): Just as humans need oxygen to live, the cells in the upstream biomanufacturing process need oxygen to survive. Oxygen levels in the human body are controlled by breathing in response to a series of delicate sensors linked to the brain. Similarly, the cells in a bioreactor are provided with a source of oxygen controlled by DO probes and a computer system in the associated DO transmitter (via the controls system of the bioreactor). DO levels that are too high or too low can cause cell death or disruption to protein production.

For mammalian cell culture, the bioreactors are aerobic. In such bioreactors, oxygen is

usually a limiting nutrient due to its low solubility in the culture media. While blending uniformity is essential for oxygen distribution in the bioreactor, bubble size distribution is the most important factor for governing mass transfer. Bubble size dictates the available interfacial area for gas-liquid mass transfer, and it is influenced by parameters such as shear rate, turbulence, and buoyancy. When bioreactors are scaled up from laboratory to production size, their design must meet both oxygen distribution and oxygen mass transfer requirements.

Volumetric oxygen transfer coefficient (k_La) is the in a culture—the transfer rate is the amount of oxygen gas that will be dissolved into the culture. It is calculated based on a number of variables in the bioreactor such as buffer, shear, bubble size, and flow rate:

- rate of oxygen transfer = Coefficient * Area * ([saturated DO concentration] – [actual DO concentration])
- saturated concentration, or the maximum amount of gas that can dissolved intothe culture
- actual concentration, or the amount of gas that is currently dissolved, which can be determined through probes or in-line measuring

The membrane electrode procedure utilizes a meter and electrode and is based on the rate at which oxygen molecules diffuse (or pass through) a membrane covering a set of electrodes. Oxygen probes have a noble metal cathode and a silver anode. Noble metals include silver, gold, or platinum. These elements are electrically connected in a potassium chloride (electrolyte) solution, separated from the sample medium by a selective gas permeable membrane. The oxygen passing through the membrane reacts with the electrolyte solution, causing a small electrical charge to develop between the electrodes. This charge can be read on a meter, and the readings correspond directly to the amount of DO present in the sample.

The physical properties of oxygen are related to temperature, so temperature measurement is also included in the probe to give temperature compensation. A constant voltage is placed across the cathode and anode. Molecular oxygen diffuses through the membrane and is reduced at the cathode by the applied voltage. This electrical process results in a current flow. The instrument detects this current, which is proportional to the partial pressure of oxygen. The Ideal Gas Law expresses the proportional relationship between gas pressure (P) and the number of gas molecules present (n):

PV = nRT

(where **P** = Pressure, **V** = Volume, **n** = moles of gas, **R** = Gas Constant,
T = Temperature in degrees Kelvin)

This relationship, along with the ability of the meter to compensate for temperature changes, allows results to be expressed as percentage oxygen or mg/L O_2.

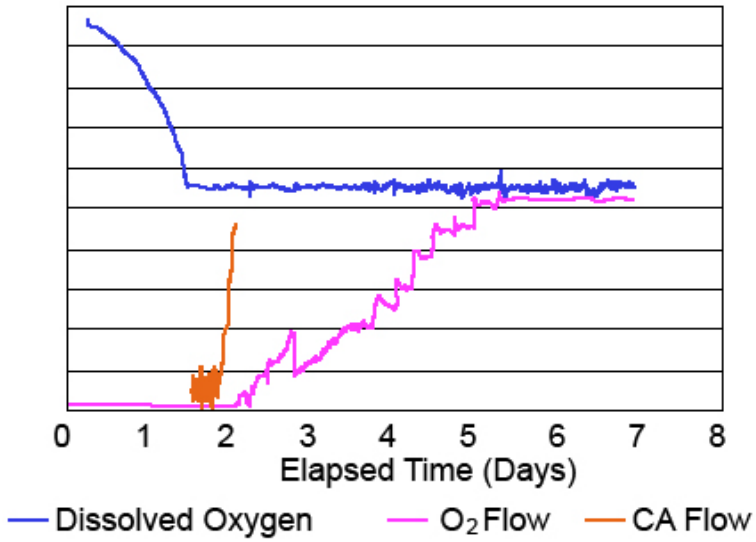


Figure 10-20. Relationship between DO value with clean air (CA) and O_2 gas supply

As the growth of the culture proceeds (lag, exponential, plateau, decline) the gas requirements change. Initially the culture in lag phase (day 0 to ~day 1.5) does not need any additional Clean Air (CA) or Oxygen. Once exponential growth (~day 1.5) starts, some CA is required to maintain the DO set point. Quickly (~Day 2) the culture requires more oxygen than can be supplied within the CA provided and the CA level is set to a capped value. The O_2 supply ramps up over time for the duration of the exponential and plateau phase (from ~day 2 to day 5) (Figure 10-20).

Cell counting

As the cells grow during the biomanufacturing process, it is necessary to keep track of cell growth. Cell counting tallies the number of viable (living) and non-viable (dead) cells and calculates a viability percentage. This percentage is the number of living cells as a percentage to the total number of cells (living and dead) in the culture. Viable cell concentration and viability are used as Forward Processing Criteria (FPC) as well as Critical Process Parameters (CPP) in the culture. Accurate and consistent cell counts are essential in creating a robust production process. The main factors to ensure accurate and consistent cell counts are obtained are mixing, dilution, and cell counting. Accuracy and precision are discussed in the ***Metrology*** chapter.

All of the methods below are off-line measurements; a sample must be taken from the bioreactor for further processing to determine cell count. When the cells are counted they are stained to distinguish between the living and dead cells as follows:

- Automated cell counting: There are a number of automated cell counting systems available that use image analysis to automate the Trypan Blue exclusion method. Examples include Vi-Cell, Cedex, and Nova units.
- Trypan Blue method: Live cells with intact cell membranes are not colored with Trypan Blue, which is not absorbed by living cells. It does, however, pass through the leaky membrane of a dead cell. Hence dead cells exhibit a distinctive blue color under a

microscope. Since live cells are excluded from staining, this staining method is also described as a Dye Exclusion Method. This method is time sensitive, and the cells must be counted as quickly as possible after staining. The Trypan Blue will begin to kill the cells over time. This method, along with the Crystal Violet method, is among the most commonly used cell counting methods. For manual counting, these are used in conjunction with a manual hemacytometer (Figure 10-21). Newer methods of non-viable cell counting use phosphatidylserine externalized by cells, which can give a more accurate measurement of cell line apoptosis.

- Crystal Violet method: This technique stains the nuclei of the cells. Viable, healthy cells either appear uniformly stained with continuous uniform edge or show mitotic structures. The Crystal Violet method is preferred for aggregating (clumpy) cell lines, as it disaggregates the clumps. However, care must be taken if the cell line has a propensity to form binucleated cells—the cell count may be higher than if Trypan Blue or another method is used.

Whether manual or automated cell counting is used, it is vital that the sample is well-mixed before drawing out any sample for dilutions. The diluted culture in the sample tube must be well mixed before drawing out a sample to fill the hemacytometer or automated unit. It is very important to load the sample immediately after making the cell suspension. Prior to loading, the hemacytometer must be clean, dry, and free of debris. Cell suspension must be evenly distributed underneath the coverslip. The area under the coverslip should be full but not flowing into the overflow well. Cells must be allowed to settle (10–20 seconds). The loaded chambers must be inspected.

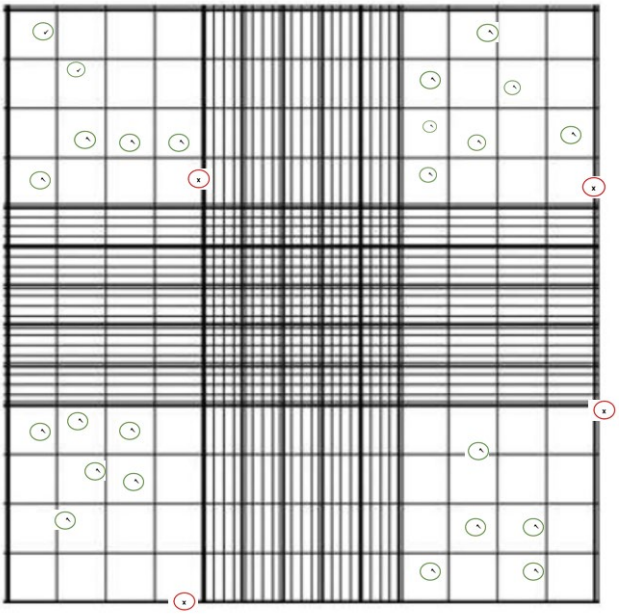


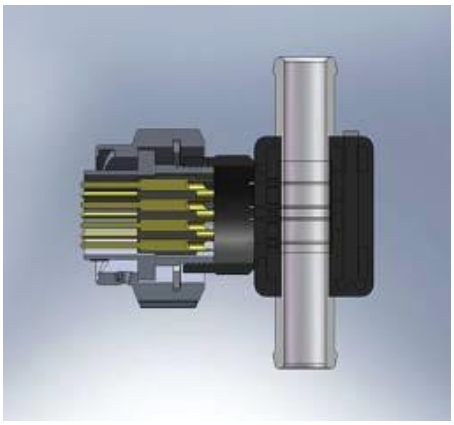
Figure 10-21 - Example hemacytometer grid – 100x magnification

Following is a method for counting cells:

1. Count cells on the top and left touching the middle line.
2. Do not count cells touching the middle line at bottom and right.
3. The viable count for each side must be >100 and <300 cells.
    - Do the cells look evenly distributed?
    - Do the cells look like they are on the same plane?
    - Are there air bubbles present?

    If the answer to any of these questions is *No*, a new slide should be prepared.

Using the biomass sensor (Figure 10-22) in-line cell counting method, only viable cells are measured. These sensors operate based on cells with intact plasma membranes (living, viable) having different capacitance (ability to store a charge) than dead cells with disrupted plasma membranes. The cells are put through an electric field; and the capacitance can be directly related to cell concentration. This method decreases the risk of contamination that can occur during sampling when using off-line counting methods. This method also voids volume loss during off-line sampling.



Source: J. Carvell- Strategies for Measuring Viable Biomass. Genetic Engineering and Biotechnology News. Vol 29. No.7 April 2009
https://www.genengnews.com/magazine/110/strategies-for-measuring-viable-biomass/

Figure 10-22 - Flow-through viable biomass probe is used with silicone tubing with disposable reactors

Packed Cell Volume (PCV) is an additional cell measurement method (Figure 10-23). The PCV method does not differentiate between dead and live cells and as such is still somewhat subjective. It is generally used to determine cell density for harvest activities.

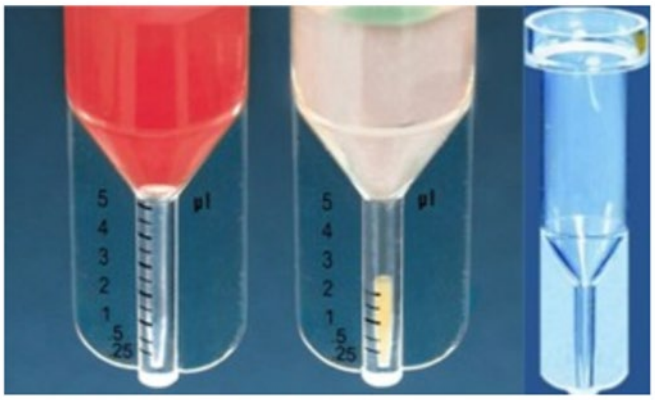


Figure 10-23: Packed Cell Volume (PCV)

Primary recovery (harvest)

The main objective of primary recovery, or harvest, is to separate the cells from the media containing the target API. It is the first step in recovery of the protein product from the culture. Each cell line has been studied, and depending on the batch record, there are certain triggers on the proper time to end the cell culture/fermentation process and initiate harvest. The decision of when these triggers are met is based upon monitoring of the culture. Samples are taken at determined intervals to monitor the progress of the culture.

Cell culture step termination is predetermined and generally based on the quality and quantity of product accumulated in the bioreactor. The highest amount of good quality product is reached when cell number drops, with cells starting to die in larger numbers. Enzymes are released from the lysed cells that could digest the product, plus the amount of cell proteins will be increasing in the conditioned media; this could put downstream processing and the entire batch at risk.

Harvest steps

The first step in mammalian harvest is usually centrifugation. **Centrifugation** involves rapid spinning of the culture from the bioreactor/fermenter (Figure 10-24). When suspended contents of a bioreactor are allowed to settle, the cells slowly sink to the bottom; and since these are the largest particles, they are the most affected by gravity. Spinning increases the force of gravity, and cells/large debris are literally spun out of the mix and discarded down a kill drain.

Microbial harvest is similar to mammalian harvest; however, a lysing step must occur prior to centrifugation to free the protein contained within the bacteria.

Figure 10-24. A centrifuge, motor and associated piping

The next step in cell harvest is the filtration step to remove large debris. The most obvious function of a filter is that of mechanical straining, or size exclusion. Particles too large to pass through the filter membrane are either trapped on the surface or within pore channels of what is called the tortuous path.

In sterile biomanufacturing, vent filters are also used to filter gases exiting from vessels. They can also be used to filter incoming gases such as CO_2, clean air, oxygen, and nitrogen required for cell culture. Gas filters are hydrophobic—they do not readily absorb water. They do, however, trap particles that may be suspended in the gas. Filters used in **clarification** of media or product are hydrophilic and readily absorb liquid.

The first filtration step in harvest is depth filtration. The large particles that do not fit through the pores are called the **retentate.** The substances small enough to pass through are found in the **filtrate**. In biomanufacturing, the API product passes through as the filtrate, along with other proteins and cell constituents small enough to pass through the filter. Some particles, while small enough to pass through a filter, get trapped due to inertial impaction. Figure 10-25 depicts a filter used in depth filtration.

Figure 10-25 A depth filter housing and internal filter stack

Depth filtration can also exploit charge difference between what is removed and the filter matrix itself. Electrokinetic adsorption results in positively-charged filter matrix components, attaching to negatively-charged items (bacteria, *Mycoplasma*, viruses, yeast, and endotoxins). These adhere to the filter and are firmly retained. The resulting porous filter structure is a tortuous network of charge-enhanced flow channels capable of removing and retaining bacteria, particulates, colloidal debris, and submicronic contaminants (hazes) to a level which mechanical straining alone cannot achieve.

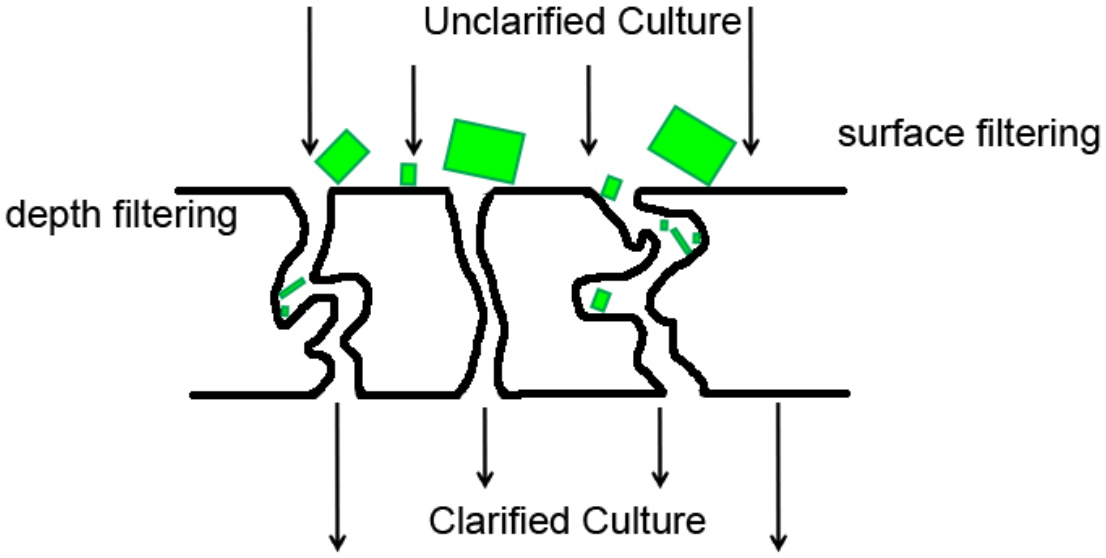


Figure 10-26. Filtration mechanics

In Figure 10-26, note the tortuous path, or channels, and mechanical straining. Some particles are retained in the channels due to inertial impaction and electrokinetic adsorption. Prior to the centrifugation and depth filtration steps, the suspension has a cloudy appearance. Both of these steps increase the translucence of the mixture, thus they are termed clarification steps. At this point the cells and larger debris have been separated from the mixture.

The next and often last step in harvesting is to perform sterile grade membrane filtration. Membrane filtration is used to remove smaller particles and potential microbial contamination. One criterion for filter performance is pore size. Pore size is most often measured in micrometers (µm or microns). Sterile grade filtration, usually rated as 0.22 µm or smaller, *removes most any bacteria. The product now has a translucent appearance and is ready to be* purified.

Filter integrity testing

To ensure filtration meets the intended purpose, filter integrity testing is performed during the biomanufacturing operation. The purpose of filter integrity testing is to ensure the integrity of the filter element(s) and the proper installation of the filter element(s). There are several types of filter integrity tests.

Pre-use integrity testing does not replace post-use testing but minimizes the chances of a post-use test failure. In most instances filters are tested post-use only. The filter is attached to a filter integrity tester device that will put the filter through pre-determined pressure tests based on the filter type. The two main types of integrity tests are bubble point and forward flow (air diffusion):

- bubble point test: measures the pressure point at which a continuous stream of air will emerge on the downstream (filtrate) side of the filter if air is applied upstream
- forward flow test: measures the rate of flow of a gas through a wetted filter; either the gas, or the downstream liquid displaced by the gas, can be measured; a passing result ensures that the filter is functioning to specification.

Check Your Knowledge

1. Chemicals that are used in CIP are usually dissolved to produce a strong base solution. An example is_____.
2. The exponential growth phase of cells is called:
   a. lag phase
   b. log phase
   c. peak phase
   d. plateau phase
3. Cells can be grown in inoculum using a variety of equipment. Depending on the type of cell line, the culture may be_____or in suspension.
4. If pH is low it means that there is a high concentration of hydrogen ions.
   a. True b. False
5. Which of the following types of agitators uses a magnet to drive the agitator?
   a. bottom mounted
   b. top mounted
   c. drive coupling
   d. multi-impeller
6. In the_____type of bioreactor, the mode of agitation is accomplished using nitrogen gas bubbling through a sparge tube within the bioreactor.
7. $k_L a$ is the volumetric oxygen transfer coefficient in a culture.
   a. True b. False
8. What is the V in the Ideal Gas Law PV = nRT
   a. velocity
   b. variable
   c. viability
   d. volume
9. In the Trypan Blue method, live cells with intact cell membranes are colored.
   a. True b. False
10. The large particles that do not fit through pores in depth filtration are called _____

Activities

1. Research disposable bioreactors and stainless steel bioreactors. Write a two-page report comparing and contrasting them, including use, construction materials, costs, benefits, drawbacks, etc.