Chapter 12

Process Development

Objectives

This chapter provides an overview of Process Development (PD) methodologies and concepts and examines how PD relates to other functions in an organization. Upon completion of this chapter student wills be able to:

- Describe process development, its role, and how it fits within the overall development and commercialization of a biopharmaceutical product
- Explain how regulations affect process development
- Define and apply common terminology
- Show how an appropriate expression system is selected
- Describe how a purification scheme is designed, including:
  - removal of process-related impurities
  - separation of product-related impurities
- State the importance of viral clearance and viral inactivation.
- Design a product stability study
- Highlight some of the key factors to consider in process scale-up
- Describe the role of PD in technology transfer and the overall tasks involved in a technology transfer project.

Terms

Analyst: the laboratory personnel performing the analytical method

Analytical method: a laboratory procedure used to analyze a sample

API: Active Pharmaceutical Ingredient

Aseptic processing: a manufacturing process that relies on maintaining a sterile environment in which the product is produced and packaged

Change Control: a formal process following a predetermined procedure set out in a Quality Assurance document or Master Validation Plan for making changes to equipment, systems, or procedures that may alter the parameters or affect expected outcomes

Downstream Processing: purification processes that typically include chromatography, viral clearance, and Tangential Flow Filtration (TFF)

Efficacy: effectiveness of the product in achieving its medicinal purpose

Expression system: protein expression consists of several steps beginning with the genetic information in DNA being transcribed, then translated into a polypeptide chain, and ultimately folding into a functional protein in its native state

Glycoproteins: proteins with specific sugar residues attached by a series of enzymatic reactions called post-translational modification

Glycosylation: oligosaccharide attached to the N of asparagine results in N-glycosylation and to the O of threonine serine and tyrosine results in O-glycosylation; N-glycosylation is common in mammalian cells; O-glycosylation is rare in humans but common in yeasts.

Identity: a test confirming the identity of a component

Potency: the specific ability or capacity of the product to produce a strong physiological or chemical effect in the body

PPs: Performance Parameters: one of a set of measurable factors, such as temperature and pressure, that define a system and determine its behavior and are varied in an experiment.

Post-translational modification: a step following protein synthesis where the polypeptide chain is modified. The most common type is the enzymatic addition of sugar residues, resulting in a glycoprotein

Process or Production Qualification (PQ): establishing confidence through appropriate testing to ensure the process is effective and reproducible

Retrospective Validation: validation of a process or piece of equipment for a product already in distribution based upon accumulated production, testing, and control data

Tangential Flow Filtration (TFF) or Cross Flow Filtration (CFF): rapid continuous technique for the separation of small molecules (salt, water) from large molecules (proteins); used for large scale buffer exchange and product concentration

Upstream Processing (USP): the first steps of production; typically includes scale-up of an identified cell line that produces the product and harvest of the product.

USP: United States Pharmacopeia

VAC: Validation Acceptance Criteria

Introduction to Process Development

Process development is performed over several years and begins when a potential drug is first expressed in a cell and is proven to have the correct **identity**. The process continues through evaluation and optimization of various steps, followed by scale-up to manufacturing. The objectives in the early phase are different from those of full scale production. The objective during the early research phase is to produce material for preliminary *in vitro* studies. The focus is not on the cost but on making material to be evaluated for biopharmaceutical production. As the product proceeds through the preclinical evaluation and human clinical trials, the focus will turn to developing a satisfactory manufacturing process.

The biomanufacturing product development process is time consuming and expensive. It is estimated to take 8–15 years, at a cost of $500 million–$1 billion, to bring a biopharmaceutical to the market, with some estimates being less conservative. The sooner the product is brought to market, the faster an organization can start recouping its research and development costs; thus a vital goal of the organization is for the final production to be cost effective and robust so that the product is profitable and the end product is safe and effective.

The process development scientist must ensure a balance between product safety, **efficacy** and keeping costs low. These are driving forces that help guide development. Thus developing a cost-effective process is a major task for a process development scientist. The objective is not to develop the perfect process since the cost and time involved would be prohibitive and too many years of development could give an organization's competitors an advantage.

The question then for process development scientists is: "What exactly has to be done, and how far do we have to go?" They must determine what is required in the early Proof of Concept (POC) phase, when the product is to be analyzed in the laboratory, and then evaluate how the requirements change during clinical manufacturing and commercial production.

The objective of process development is to develop a robust, scalable, reproducible, and cost-effective process that results in safe and efficacious biopharmaceuticals. This chapter will examine some of factors that can potentially impact this objective.

Choice of Expression System

The two main criteria to be considered when choosing an expression system are:

- **protein structure**: whether or not it has a post-translational modification
- **cost of goods**: the cost of different systems can vary greatly

Before choosing from one of the commonly used expression systems, however, a proper understanding of post-translational modification (PTM) is needed.

The primary structure (amino acid sequence) determines the identity of a protein product. Modification of an amino acid of the polypeptide chain is defined as PTM. The modifications can be the addition of lipid or oligosaccharide side components resulting in a lipoprotein or glycoprotein respectively. Other chemical modifications of certain amino acids of the polypeptide chain can occur, such as oxidation, de-amination, cross-linking, etc.

The most important of these modifications is glycosylation. After the polypeptide is produced in the cells, a series of enzymes add specific sugar moieties (glycosyl residues) to certain amino acid side chains. The result is a glycoprotein (a protein with sugar). Approximately 70 percent of human proteins are glycosylated. In many of the glycoproteins the biological activity of the parent protein is highly affected. Thus the question of glycosylation ability is crucial when choosing an expression system.

With a clear understanding of glycosylation and the role it plays in PTM, one is now prepared to examine the various expression systems available.

Bacterial systems are attractive, as they are inexpensive, but these systems are unable to perform post-translational glycosylation. Bacterial systems can be used for products that do not require glycosylation for biological activity. Yeast cultures are also inexpensive but add non-human glycans, which have been found to be immunogenic.

Insect systems occupy the space between yeast and mammalian expression, both in cost and characteristics of the product. Presently, large scale production via insect systems is not common, with most used mainly for research purposes. Mammalian expression systems, though they are expensive and complicated, are widely used. The most popular cells are Chinese Hamster Ovary cells (CHO) and Non-Immunoglobulin Secreting mouse myeloma cells (NSO), both of which result in products most closely mimicking human post translational modification (PTM).

Transgenic systems have existed for many years, and several technologies have been developed for the expression of recombinant proteins in both animals and plants. Two advantages of this type of expression are a favorable cost and the flexibility and simplicity of scale-up of the living bioreactors. Presently, however, the only product approved by the European Medicines Agency (EMA) and FDA is a recombinant antithrombin, ATryn®, expressed in the milk of transgenic goats.

As compared to the typical human forms, the transgenic animal and plant systems produce glycoproteins with somewhat different glycosylation patterns, structures, and compositions. Furthermore, plants add xylose—a 5-carbon sugar that humans do not use in protein glycosylation and which can trigger unwanted side effects. Regulatory concern with plants relates to the containment of the genetically-modified organism (GMO, the modified plant) and possible spreading of drug-producing plants outside of the designated production field. The final decision is still pending, however, as scientists have genetically altered plants to eliminate the undesirable xylose addition.

Stages of Process Development

Based on the characteristics of the work performed, the typical stages of process development can be identified as either *early*, *mid*, or *late*.

Early stage involves research and the goal is to express a recombinant protein with a well-defined biological activity. During these activities, the objective is to prove that the expressed protein contains the correct sequence of amino acids. Any cell culture and purification method is acceptable for the experimental proof of principle. Solid science and data recording must be

performed. No regulatory restrictions apply at this stage.

Mid-stage is the stage in which most of the development occurs and can last several years. During this stage analytical tools are developed to assess the result of the development activities. Analytical tools must address:

- biological activity
- product identity
- product purity
- product and process-related impurities

Product impurities include truncated and aggregated product, incorrect disulfide formation, and other chemical changes of certain amino acid residues. Process-related impurities include host cell proteins and growth medium or buffer components that might be potentially hazardous if present in the final product. To assess all of these, assays have to be available for process development. Proper analytical tools are crucial for the success of development.

The main objective during this stage is to provide a safe and efficacious product using a process that is robust and cost-effective. The activities that address these issues are the **process characterization** and **process validation** studies. The former are used to establish the ranges of various parameters, and the latter are performed in a more regulated manner, typically either in the laboratory and/or at the manufacturing scale. Together these studies define the range of each process parameter within which the process can be safely performed. The same rule applies to all process variables of both the upstream and downstream steps.

The following example illustrates how important the range of process parameters and process variables can be:

> Prepare a buffer, pH = 7.3. Suppose the pH of the prepared buffer comes out as pH 7.4. What happens? If it was prepared in the lab and the pH is 7.4 rather than 7.3, it can be easily redone. However, when making 10,000 L of the same buffer, the cost and labor invested is considerable. Making the WFI for it, releasing the buffer components in the QC lab, weighing out the large quantities of salt components, making up the solution, filtering the 10,000 L solution aseptically—time and money that is wasted if the buffer is not at the desired pH.
>
> What can be done? During process development, the acceptable range for a process parameter is defined. In this example, assume the acceptable pH range is 7.2–7.4. The same process step is performed with buffer made up at pH 7.2 and at pH 7.4. If the result supports that the process can handle small changes, we can use the buffer at pH 7.4. With small studies we can reduce waste at the manufacturing site. This is a robust procedure.

Most of the activities in the mid-stage occur during the years when a project undergoes preclinical and clinical evaluation. As the clinical trials proceed, the project matures and the regulatory requirements become stricter.

While the process is scaled up and further optimized, documentation is prepared. Standard Operating Procedures (SOPs) and Batch Records are written and approved. In some production

lines, as many as six hundred Standard Operating Procedures are necessary to guarantee the quality and safety of the desired product. Documents for the Quality System are also written during this stage.

Late stage begins with the filing of the **Biologics License Agreement (BLA)**. Preparations are made to begin the Clinical Phase III study using the process as it currently stands. Process validation begins after process development is complete and process characterization studies have been performed. Knowledge of the process is crucial prior to the start of process validation studies. The studies are outlined in the Validation Master Plan, which is reviewed and approved prior to the initiation of the validation studies. An additional set of studies that must be completed prior to process validation is the **analytical assay validation**. Furthermore, so as to not compromise the data generated during process validation, the critical analytical methods used must likewise be validated. Assays, process steps, equipment, and software are validated during the late stage.

After validation is complete, any additional modifications to the process will need to be performed using **Change Control** methodology. Change Control requires a plan that describes the objective of a process modification, the expected outcome, and the assessment as to whether a change in product quality is possible. Modifications of the process in any manner during this phase is highly regulated.

Process characterization

Process characterization involves performing bench-scale studies to demonstrate **process robustness** and help predict the performance of the process within the constraints of the operational ranges to be used. **Operating Parameters (OPs)** are identified that are most likely to impact the **Process Performance Parameters (PPs)**.

Process characterization also involves preliminary studies such as solution holding studies (growth medium and buffer) and intermediate holding studies. These studies are indicative of the potential weak points of a process and serve as screening studies for later validation studies. For example, during a manufacturing process that operates continuously (twenty-four hours per day), it is important to know how long process fluids can be stored. The intermediates contain proteins that are unstable. There may be proteases present, which could break down proteins. Additionally, pH changes can trigger aggregation. These are reasons why quick studies are designed to assess characteristics such as duration and temperature concerning the intermediate material. Simple studies are designed to ensure comparable results are reached when repeating the process step (e.g., leaving the feed stream at ambient temperature for a day or two or storing it at 2–8°C).

Observations such as these can later be referenced as an initial hold study. These studies are necessary to prepare for unexpected issues during manufacturing, such as a power outage or equipment failure that could stop the process. Unless there are supporting data that processing can be held for the desired time period without risking the quality of the product, processing cannot resume even after correcting the issue. The result would be the loss of the production batch.

Typical conditions are **ambient temperature** and frozen conditions (2–8°C). For salt solutions, including buffer and sanitizing agents, the general approach is to study the buffer's pH and ionic strength for the desired time range for both ambient temperature and cold storage temperature. Table 12-1 is an example of a buffer hold study.

Table 12-1. Example buffer hold study

2–8° C

Test	Day 0	Week 1	Week 2	Week 4	Week 8
pH	√	√	√	√	√
conductivity	√	√	√	√	√

Ambient Temperature

Test	Day 0	Day 1	Day 3	Week 1	Week 2	Week 4
pH	√	√	√	√	√	√
conductivity	√	√	√	√	√	√

In-process samples containing the protein of interest will have a different study design as the focus will be on the integrity of the product. The characteristics of a biochemical study are **aggregation**, **degradation**, and **charge modifications**. Aggregation is assessed by Size Exclusion Chromatography (SEC); degradation can be analyzed by SDS-PAGE under reducing and non-reducing conditions (R/NR SDS-PAGE); and isoform analysis is typically performed using Iso Electric Focusing (IEF), separating the components by means of their isoelectric points. The study will always include biological characterization using at least one biological activity assay. The temperature studies will include freezing. Table 12-2 is an example of an in-process hold study.

Table 12-2. Example in-process hold study

Ambient Temperature

Test	0 hr	2 hr	4 hr	12 hr
aggregation	√	√	√	√
degradation	√	√	√	√
isoform	√	√	√	√
bioassay	√	√	√	√

$2–8^0$ C

Test	Day 0	Day 1	Day 2	Day 5	Day 7
aggregation	√	√	√	√	√
degradation	√	√	√	√	√
isoform	√	√	√	√	√
bioassay	√	√	√	√	√

-40^0 C Frozen Storage

Test	Day 0	Week 1	Month 1	Month 3	Month 6	Month 12
aggregation	√	√	√	√	√	√
degradation	√	√	√	√	√	
isoform	√	√	√	√	√	
bioassay	√	√	√	√	√	√

Analysis of the data will determine how the material will be stored. The general aim is to be able to store solutions for 1–4 weeks. If the data suggests that the solution quality is not acceptable when stored at ambient temperature, the solution will have to be stored chilled. Therefore, cold storage data is the most common data required for analysis. Storage at ambient temperature is performed to cover potential emergency situations.

Temperature will be chosen where product quality is not compromised—product is not aggregating (SEC) or breaking down (R/NR SDS-PAGE) during storage. Isoform analysis is a good indicator of many structural changes.

For product that contains in-process material, the storage time cannot be extended. The process stream can easily degrade and become a potential food source for bacteria.

Process validation

Process validation provides documented evidence that a given process, operated within established parameters, can effectively and reproducibly produce an Active Pharmaceutical Ingredient (API) meeting pre-determined acceptance criteria. While final drug products must meet specifications based on standards guided by safety concerns, intermediate process steps do not have equally strict criteria. However, they still must meet a number of acceptance criteria to demonstrate process consistency.

Establishing appropriate **Validation Acceptance Criteria (VAC)** is one of the greatest challenges in the development of a commercial biopharmaceutical manufacturing process. Setting VAC that are too broad does not provide sufficient process control and VAC that are too narrow can result in failed runs even though the process may be performing adequately. Traditional process validation consists of nine runs for the validation of a parameter. The process is tested at the low, mid, and high point for each parameter, with each point tested three times. For example, the media acceptable buffer pH range is pH 7.2–7.6. The cell culture is done three times at each of the following points: pH 7.2, pH7.4, and pH7.6.

Some of the validation protocols can be performed on a qualified small scale system. Following is a summary of the **small scale studies**:

- **growth media and buffer stability (chemical and/or biochemical)** are used to collect data on how long the components of a solution are stable (microbial stability studies are performed in the actual manufacturing vessels).
- **initial resin and membrane reuse and hold studies:** are used to assess how long the chromatography resins and cross-flow membranes can be stored as well as how many cycles of production these matrices can take without loss of performance. The results are captured in the manufacturing batch records. The studies will be repeated at full scale when data are collected during manufacturing runs.
- **process- and product-related impurities:**
  - clearance studies:
    - host cell DNA (*E. coli*, NS0, CHO transgenic host)
    - endotoxins (originated from Gram-negative bacteria)
    - viral clearance (potential risk, not applicable to bacterial and yeast expression system)
  - removal studies:

- certain media components which could be carried through the process and might be harmful to patients
- leaching protein ligands from resin (e.g., protein A leaching from the affinity resin widely used to purify antibodies) could interact with antibodies of the patient if they got into the final vial
- leachables, extractables from single-use filters and media bags, as some of the components might be toxic
- hazardous chemicals used in the process- an example is acetonitrile used for reversed-phase chromatography.

– product-related impurities, which can be immunogenic, affect biological activity and or influence the clearance of the product. The versions of the product include those that are:

- truncated
- aggregated
- modified either within the cell or during the process by other mechanisms

Full scale studies include:

- **media and buffer storage expiration studies**, assessing bioburden in the actual storage vessels
- **resin and membrane reuse and cleaning/storage studies** at the manufacturing scale, which typically confirm data from small scale predictive studies

Viral Inactivation and Viral Clearance

A potential risk associated with the production and use of biological products is viral contamination. This contamination may either be present in the source material (e.g. human or animal tissues and cell banks) or introduced in the manufacturing process through the use of products of animal origin in cell culture (e.g. fetal calf serum, trypsin, etc.). Therefore, studies must cover all known and potentially unknown types of viruses (single or double stranded DNA and RNA viruses).

Viral inactivation studies are performed to find conditions that will inhibit virus reproduction while leaving the product unharmed. The methods include pH adjustment or solvent/detergent treatment, each of which affect the protein or lipid coat of the virus particles. Ideally,the objectives of **viral clearance validation** studies are to establish, both qualitatively and quantitatively, the overall level of virus clearance. Evidence of viral clearance must be obtained in all stages of purification, and adequate viral removal and/or inactivation must be proven. The method used to validate viral removal and/or inactivation is to challenge the system through the deliberate addition (spiking) of significant amounts of virus into both the crude material to be purified and the different fractions obtained during the various purification stages. Removal or inactivation of the virus during the subsequent stages of purification and/or inactivation is thereafter determined.

The Impact of Process Development on Upstream and Downstream Processing

During process development, protein separation is not the only objective. Developing a good, cost-effective process is also necessary. A good process must include scalable, reproducible, and robust steps. Components must be stable for many cycles of processing, cleaning, and sanitization without a noticeable loss of performance. Upstream and downstream efforts are examined in the following sections, as they are greatly impacted by the outcome of process development efforts.

Upstream Processing

As briefly discussed earlier, the first decision in the development of a product is the choice of the drug candidate. The second involves the selection of the expression system. The primary objective is to produce a biologically active product. The API must be safe and the processing cost-effective.

Expression systems

The oldest known fermentation system uses yeast and the most familiar fermentation processes are making bread with baker's yeast (*Saccharomyces cerevisiae*) or fermenting malted barley into beer (using *S. cerevisiae or S. pastorianus*). Yeast production is fast, inexpensive, and a cost-effective system. Unfortunately, yeast attaches carbohydrates on unusual locations and tends to over decorate (overglycosylate) the peptide chain and the resulting products have undesirable side-effects. As a result, organizations are working on re-engineering yeast enzymes to produce predictable and acceptable **glycoproteins**. The thought is *Pichia pastoris* yeast can be "humanized" so that the genetically engineered yeast would have a **glycosylation** pattern similar to what human cells would produce.

Prokaryotic expression most widely uses *E. coli*, a Gram-negative host cell, for recombinant DNA work. In most cases, the bacterial cell wall has to be disrupted to recover the product from the cell. The expressed product can be soluble or insoluble in the cell. If the product is expressed in the cytoplasm and is insoluble, inclusion bodies are formed. The inclusion bodies are nuggets of inactive proteins, misfolded and aggregated, often through cross-linking of cysteine residues. The inclusion bodies contain highly concentrated product, with purity often over 90 percent. Thus purification can be rather easy, as compared to brute-force approaches generally used in biomanufacturing. However, the material is in an inactive state (non-native). To bring the protein into a correctly-folded state (native), the inactive, non-native protein has to be treated with reducing agents to eliminate disulfide cross-links. The reduced and non-native protein can then be carefully refolded under tightly controlled conditions, resulting in biologically-active material. Refolding can be a challenging step in this approach. In cases where the expression results in soluble protein, refolding is not necessary but purity will be considerably lower. Processing principles are similar to those used with any other expression system.

Although many simpler proteins can be produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified) can be successfully expressed in animal or human cells (**mammalian expression**). The cost of growing mammalian cell cultures is high, but oftentimes this is the only way the desired biopharmaceutical can be produced. Mammalian expression is applied in the production of NS0, many vaccines, hormones, antibodies, and native/modified proteins and fusion proteins. The most commonly used cell line is Chinese Hamster Ovary (CHO) cells. Cells are grown and maintained at an appropriate temperature and gas mixture. Typically for mammalian cells, a temperature of 37°C is used and the $NaHCO_3$ containing media buffered with 5% CO_2 in an incubator (CO_2 incubator). Culture conditions can vary widely for each cell type and variation of conditions for a particular cell type can result in different phenotypes being expressed.

Another commonly varied factor in culture systems is the growth medium. Composition of growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. The growth factors used to supplement media are often derived from animal blood, such as calf serum. One complication of these blood-derived ingredients, however, is the potential for contamination of the culture with viruses or prions. Therefore, the current practice is to minimize or eliminate the use of components of animal origin. Alternative strategies involve sourcing the animal blood from countries with minimum BSE/TSE risk, such as Australia and New Zealand. Additionally, purified nutrient concentrates can be used, which are derived from serum in place of whole animal serum for cell culture. In the current technologies, serum components are only used in very early cell line development work. Once a cell line goes through clonal selection, media without animal products is used.

Cells can be grown in **suspension** or in **adherent** cultures. Some cells live naturally in suspension without being attached to a surface, such as cells in the bloodstream. There are also cell lines that have been modified to survive in suspension cultures so that they can be grown to a higher density than adherent conditions would allow. Adherent cells require a surface, such as tissue culture plastic or microcarrier, which may be coated to increase adhesion properties. Most cells derived from solid tissues are adherent. When expression stability is established, the recombinant cell line is tested to ensure the cell line is acceptable and contains no adventitious agents. The cell line that meets all acceptance criteria will be used in the **Master Cell Bank (MCB)**, and cells will be stored in liquid nitrogen (-196 °C) under tightly controlled conditions.

Vials are taken and expanded into the Working Cell Bank (WCB) for cGMP production. Cells from the WCB typically contain 1 mL of cell suspension. Cells from the WCB are grown by multiple scale-up steps from small stirred flasks through a train of seed bioreactors of increasing size up to production bioreactor. The production bioreactor may have a volume of 5000–20,000 L, depending on product requirements.

Bioreactors can be classified as:

- **stirred**: mechanical mixing and air-sparged liquid media
- **airlift**: uses air sparging to mix the media
- **membrane**, **hollow fiber**: cells are separated from the nutrients by semi-permeable barrier membranes. The permeability is selected so as to allow the product, nutrients,

and various breakdown products to pass through the membranes while the cells are retained by the semi-permeable membrane.

Several organizations have been working on the development of large single-use bioreactors. Smaller scale units (up to 200 L) have been utilized in many laboratories and manufacturing facilities. Recently, 1000 L working volume systems have been introduced. These single use units can be cost-effective systems for manufacturing as well as for supplying material for early phase clinical trials.

Scale-up considerations

Successful scale-up means a shortened cycle from research and development to full-scale production. Development occurs at small scale (bench scale), which can range from tens or hundreds of milliliters of cell culture media to liter sizes. Similarly, the filtration and chromatography step development may start with just a few milliters of process fluid purified on 1–5 mL columns. Syringe filters can be used. The scale between bench and manufacturing is pilot scale. At commercial manufacturing scales (large scale production), the production bioreactor size can be up to 20,000 L of working volume, and the chromatography column size can be hundreds of liters.

Initially cell culture conditions are optimized by the cell culture group. The downstream process is developed by the purification scientists. The analytical staff performs assays as requested and provides the results. Based on these results, the process is further developed. These groups typically belong to a Research and Development (R&D) department.

Later a process called technology transfer occurs. Technology transfer includes:

- upstream and downstream process transfer
- assay development and transfer to the QC group
- identification, purchasing, testing of all chemicals, growth medium, chromatography media, and filters

The upstream and downstream process is coupled and run at bench scale on a liter scale bioreactor with scaled purification and filtration equipment. This exercise is repeated, with the scale increased each time. Throughout the scale-up period a multidisciplinary team, usually headed by the project manager, works through all aspects of the series of tasks. Practically all departments of the organization participate in technology transfer.

QA and Regulatory Affairs provide guidance during the process of moving the product from R&D to the cGMP environment. As the scale grows larger, engineering becomes heavily involved. It should be emphasized that scale-up is not a trivial matter. For many smaller companies, scale-up can be one of the most complicating elements of the development of a biotherapeutic.

The steps become critical when the project reaches the pilot plant. The pilot plant is a small version of the actual manufacturing plant. The objective of having a pilot plant is to gain experience in operating the actual plant. All pilot run records are set up such that they could easily be turned into SOPs and batch records for the actual manufacturing plant.

Scale-up would be very simple if all parameters affecting cells remained the same. However, special circumstances must be considered. Potential issues include heat transfer, nutrient depletion (particularly O_2 depletion) and the build-up of toxic by-products (such as ammonia and lactic acid), and the removal of CO_2. Numerous empirical and semi-empirical relationships are often used to correlate variables like shear rates and oxygen mass transfer with physical parameters such as impeller speed and reactor dimensions.

Harvest

The objective of the harvest step is the removal of cells and cell debris to enable product capture on the first chromatography column. Cell harvest timing is critical for both quantity and quality of the product. The longer the culture can grow, the higher the amount of product accumulated in the batch. This would suggest keeping the culture going for a long period of time. However, when cells begin to die, the released enzymes can harm the product. The generally applied technique is to determine Dissolved Oxygen (DO) and cell counts for the assessment of cell density. Quality of product has to be assessed regularly through the latter part of the production phase in mammalian cell culture systems.

Centrifugation uses centrifugal force to accelerate the settling of the solids that would normally occur during sedimentation. Most industrial applications use disk stack centrifuges to remove cells and cell debris. Disk stack centrifuges operate continuously, making throughput consistent and controlling the time for the harvest step. Filtration techniques, as described in ***Chapter 10 Upstream Processing*** and ***Chapter 11 Downstream Processing***, are also used during harvest. Filtration offers two advantages over centrifugation: 1) the harvest stream is particle free and 2) the process is gentle on the cells. Filtration is a more sophisticated technique than centrifugation.

Downstream Processing

A major part of the development effort for biochemical processes is spent on the optimization of media and productivity of the cell line. However, the costs incurred by downstream processes often determine the profitability of the production process. Downstream processing consists of two types of separation methods—chromatography and filtration. The main purpose of chromatography is the separation of the product from other large molecules (e.g., separating other proteins from degraded cells, host cell DNA, endotoxins, and potential viruses). One filtration technique, **Tangential Flow Filtration (TFF),** is widely used for the separation of small molecules, such as salts (average MW 50–200) and water from proteins (average MW 100,000). TFF uses a semi-permeable membrane that allows small molecules to pass through while retaining larger molecules. TFF membranes can have variable pore sizes that determine the limit of the size of the molecules that can pass through the membrane.

Downstream processing also includes **buffer exchange/concentration** and **protein purification** steps. In the case of buffer exchange, small molecules (e.g., salts, metabolites, etc.) are removed from the sample and replaced with a new buffer. During the concentration of a protein solution, water is removed using semi-permeable membrane. The composition of the salt solution or buffer does not change.

Methods of protein purification include:

- **Precipitation**: Precipitation is the oldest technique applied in protein purification. Protein separation is achieved by varying the salt concentration, pH, and temperature. As the salt concentration increases, proteins precipitate. Since the salt concentration where a protein falls out of solution is characteristic to the protein, this technique can be used to separate proteins. A commonly used salt is ammonium sulfate. Another precipitating agent is cold ethanol for blood plasma protein separation. This type of precipitation, called the Cohn Process is inexpensive and scalable and has been widely used since World War II.
- **Crystallization**: Like small molecules, many proteins can be crystallized but often with great difficulty initially. Some crystallizing agents work well, others do not work well with the carefully defined processes, and others do not meet regulatory requirements. Crystallization development for r-protein purification requires a good understanding of the technique and the precipitating agents used. Separation can be highly selective, inexpensive, and scalable in expert hands.
- **High Performance Tangential Flow Filtration (HPTFF)**: This method is rarely used, even though the results are impressive. The gel layer formed on a membrane during filtration depends on transmembrane pressure and is also affected by salt concentration and pH. By varying these parameters, HPTFF can result in great selectivity. The technology is inexpensive and scalable in expert hands.
- **Chromatography**: This method is the most widely used technique. There are several modes of chromatography, explained in earlier chapters.

  The scaling-up of chromatography steps includes keeping these parameters constant:

  - residence time or linear velocity
  - the height of the chromatography bed; therefore, during scale-up the diameter of the column will be increasing
  - buffer conditions (pH, salt concentration)
  - collection volume (expressed in column volume)
  - column characteristics (peak asymmetry, theoretical plate height)

  An example of a scale-up would be as follows:

  For a small scale process, the capture column is 1x30, meaning a 1 cm diameter and a 30 cm bed height and the volume is 23.55 mL. The manufacturing scale capture column has a 2 m diameter and a 30 cm bed height and the volume is 942 L. If a five column volume of buffer is needed to equilibrate, the buffer requirement for bench scale is 117.75 mL and for large scale is 4170 L.

  Engineering needs increase because of the scale-up, as larger quantities of salts must be transported and weighed. Lifting the top of the 942 L stainless steel column requires

mechanical assistance. Buffer containers will be hard piped to the column, as well as to the filters.

Check Your Knowledge

1. Define the following key terms:
    a. post-translational modification
    b. downstream processing
    c. upstream processing
2. The objective of process development is to develop a robust, scalable, reproducible, and cost-effective process that results in _____ and _____ biopharmaceuticals.
3. In many of the glycoproteins, the _____ is highly affected by the carbohydrate structure on the molecule.
4. The _____ are nuggets of misfolded, inactive protein aggregates.
5. Which of the following systems qualifies as a mammalian expression system? (select all that apply)
    a. *E. coli*
    b. human cells
    c. *Saccharomyces cerevisiae*
    d. transgenic goat
    e. transgenic tobacco
    f. NS0 cells
    g. CHO cells
6. Which process step can be performed by centrifugation of cross-flow filtration?
7. Which step consists of purification and buffer exchange?
8. _____ relies on the reversible charge-charge interactions between the proteins in a sample and the charges immobilized on the resin.
9. Which chromatographic separation method listed below is based on each of the following?
    a. size _____
    b. charge _____
    c. biological recognition _____
    d. hydrophobic interaction_____

Activities

1. Select an expression system (bacterial, mammalian, yeast, insect, transgenic) and create a PowerPoint presentation describing how it works, its application, benefits, and drawbacks. Share the presentation with your classmates.
2. Select one of the following: aggregation, degradation, or charge modification. Research the corresponding analysis type (i.e., SEC for aggregation, SDS-PAGE for degradation).

Write a two-page paper on the analysis process, describing how it is performed and the equipment used.