1 Introduction to Biopharmaceutical Processes

1.1 Context

Thanks to major progress in the understanding of the biological processes involved in human diseases, protein-based drugs have emerged as an important class of therapeutics in the 1980s [1, 2]. The main asset of proteins is rooted in their ability to perform highly specific and complex sets of biological functions that can hardly be mimicked by traditional chemical drugs [2, 3]. The development and commercialization of protein-based drugs have been fostered by significant advances in protein and genetic engineering to such an extent that they are nowadays mostly produced by living organisms [1, 4, 5]. To name just a few examples, therapeutic monoclonal antibodies, hormones, and blood factors are produced at the commercial scale using the so-called *recombinant technology*. Besides recombinant proteins, a few proteins are still extracted from their native source, such as pancreatic enzymes or plasma proteins [2]. A third strategy to produce protein-based drugs is chemical synthesis, which is nevertheless limited to rather small proteins and peptides [2, 6]. Therapeutic peptides represent a kind of hybrid class of drugs, which bridge the gap between small-molecule and protein drugs in terms of physical properties, clinical applications, and means of production [3]. In the following, they will be placed under the umbrella "therapeutic proteins" due to their amino acid content. In any production process, whether based on the recombinant technology, on the extraction of a protein from its native source, or on chemical synthesis, the target protein has to be isolated from a complex mixture, and efficient purification processes are crucial to reach the high purity grades required for medical use.

In this chapter, we provide a succinct description of typical biopharmaceutical processes in order to contextualize the three main aspects that will be addressed in this book: chromatography, protein conjugation, and protein aggregation. In a first part, we briefly present the main unit operations encountered in the production of therapeutic proteins, and then we discuss *if* and *how* the biopharmaceutical industry could benefit from the use of continuous technologies.

1.2 Single-Unit Operations

Biopharmaceutical production processes are commonly divided into two parts: *upstream processing*, where the protein is produced by living organisms, and *downstream*

processing, where a series of purification steps are performed to meet certain purity specifications. Although the terms *upstream* and *downstream* are usually reserved for the production of recombinant proteins, they could in principle be extended to synthetic proteins, where the term *upstream* would designate the chemical synthesis step.

There exist a wide variety of biopharmaceutical production processes, which greatly differ both in their upstream and downstream parts. Over the past years, there were some attempts to develop a universal protein purification platform [7, 8]. However, the number of available protein sequences rises dramatically with time, and protein databases now contain more than 20 million different amino acid sequences [9]. The classification of these proteins into comprehensive domain families led to the identification of about 16,000 different families, among which about two-thirds are singletons (i.e., single-member families) [10, 11]. Because the choice of the purification process is strongly dictated by the protein structure, this undoubtedly annihilates the vision of a universal purification platform. It is thus impossible to describe here a general production process applicable to any therapeutic protein.

Nevertheless, for some families of proteins, it is possible to identify rather general production platforms, as it is the case for monoclonal antibodies (mAbs) [12]. This is related to the fact that mAbs all share a common structure, with one constant domain (Fc) that is identical within a class of immunoglobulin (Ig) and two antigen-binding domains (Fab) that are specific to the antibody of interest. Besides their industrial relevance [13], mAbs therefore represent a good didactic example to introduce general concepts about biopharmaceutical processes in general and about downstream processes in particular, which is at the core of this book. Accordingly, we will take mAbs as a reference in the following. A representative example of a mAb production process is represented in Figure 1.1, and we will largely follow this scheme throughout this section. Deviations from this general scheme will be pointed out in the text, and appropriate references for nonantibody proteins will be indicated when possible.

1.2.1 Cell Culture

As mentioned earlier, a great number of tailor-made therapeutic proteins are nowadays produced by living organisms. Recombinant proteins can be expressed in a wide variety of systems including bacteria, mammalian cells, yeasts, fungi, as well as transgenic plants and animals [5].

Among these systems, *Escherichia coli* (*E. coli*) bacteria are particularly attractive due to their well-characterized genetics, great versatility, rapid growth, low media cost, and high expression level [14]. However, *E. coli* bacteria suffer from the inability to perform complex posttranslational modifications, such as glycosylation, and from the difficulty to achieve proteolytic protein maturation and disulfide bond formation. Moreover, proteins expressed by *E. coli* are often produced as inclusion bodies, which are intracellular insoluble deposits containing the protein at high concentration in an inactive aggregated state. In this case, it is necessary to dissolve these inclusion bodies and to properly refold the protein of interest *in vitro*, which may turn out to be a very challenging operation [15].

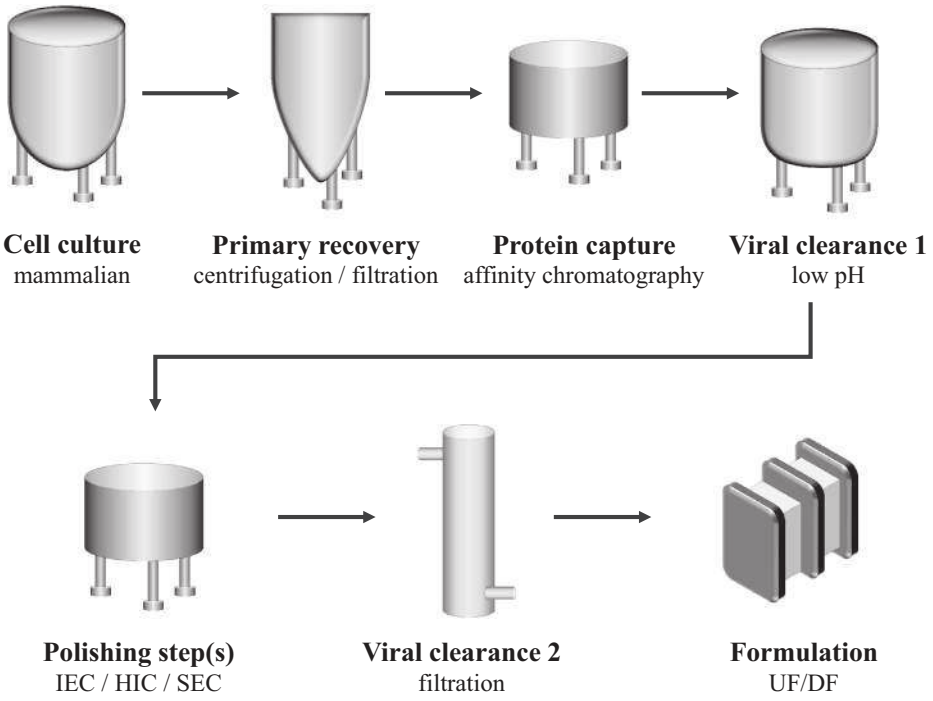

Figure 1.1 Schematic example of a process for the manufacturing of mAbs.

The aforementioned limitations prohibit bacteria from expressing some pharmaceutically relevant proteins, such as mAbs. The latter are largely produced by mammalian cell culture, and in particular with Chinese hamster ovary (CHO) cells [4]. Despite their high cost and complex genetics, mammalian cells are the system of choice for expressing large proteins (above 100 kDa) whose biological activity is strongly affected by both folding and posttranslational modifications [16].

Cell culture falls out of the scope of this book, and the reader interested in biochemical engineering is referred to the following references [17–19].

1.2.2 Primary Recovery

After the cell culture step, the protein of interest is retrieved either by collecting the culture broth supernatant (if the protein is expressed extracellularly) or after supernatant removal and cell disruption (if the protein is expressed intracellularly). In both cases, the first step consists in separating the supernatant from the cells and cell debris formed during cell autolysis, which always occurs to some extent during the cultivation step. This solid–liquid separation step is usually performed with centrifugation or filtration [20, 21] and is relatively simple due to the large size of cells and cell debris as compared to proteins.

In the case where the supernatant is collected and the cells are discarded, as it is typically the case for mammalian cell culture, this first purification step is termed *clarification*, and the recovered liquid is usually referred to as the *harvested cell culture fluid*.

1.2.3 Protein Capture

The fluid obtained after primary recovery contains a wide range of impurities, which include components secreted by the living cells and intracellular components released during cell lysis (e.g., proteins, nucleic acids, lipids, and endotoxins), unused components from the cell culture media (e.g., salts, sugars, amino acids, surfactants, growth factors), and viruses that potentially infected the cells [21]. Among all these species, endotoxins and viruses are extremely hazardous and thus need to be thoroughly eliminated.

At this level, the objective is to extract the maximum amount of target protein from the biological feedstock and to remove as much as possible of the aforementioned impurities (although not necessarily satisfying the final purity requirement). Appropriately, this step is commonly called the *protein capture step*. During this operation, it is also particularly important to recover the protein at a sufficiently high concentration in order to reduce the volume to be treated during the subsequent purification steps. One of the most powerful techniques to perform protein capture is certainly affinity chromatography, which relies on the highly specific reversible interaction between the protein of interest and a natural or synthetic ligand covalently linked on the solid support. The majority of antibody purification platforms employ Protein A as affinity ligand, which interacts specifically with the Fc domain of immunoglobulin G (IgG) [21–23]. Other specific ligands include antibodies, which are used in the industrial purification of recombinant factor VIII for instance [24], heparin that is widely employed in the purification of plasma proteins [25], as well as substrates, co-factors, and inhibitors that are typically selected for enzyme purification [26]. The high price of affinity chromatographic media promotes the development of alternative methods to perform the capture step [22, 27], and two notable exceptions of antibody purifications using ion exchange chromatography are Zenapax® (Daclizumab) and Humira® (Adalimumab) [22]. More details about affinity chromatography and other types of chromatography will be given in Chapter 2, while multicolumn chromatographic processes relevant for the capture step will be presented in Chapter 4.

Although protein chromatography is traditionally performed with packed beds, membrane chromatography has also been examined as an alternative [20, 28–31]. In addition, aqueous two-phase partitioning, which was popular in the 1980s for enzyme purification [32], has recently regained substantial interest in the biotechnology industry as a potential strategy to perform the capture step [20, 33–35]. Finally, precipitation, either of the impurities or of the protein of interest, has often been used as a first step in protein purification processes [20, 36–38]. A noteworthy example among mAbs is Orthoclone OKT®3 (Muromomab CD3), which is recovered from the clarified supernatant by ammonium sulfate induced precipitation [22].

1.2.4 Polishing Steps

After the capture step, a number of *polishing steps* aim at removing the residual impurities in order to obtain the product at the required purity. The expression "intermediate purification steps" is sometimes used to reserve the term *polishing* to the very last purification operation. In this case, the purification process is divided into three parts: a capture step, one or several intermediate purification steps, and a polishing step. There is nevertheless no conceptual difference between intermediate and polishing steps, and we will therefore use the term *polishing* to denote any purification step subsequent to the capture step.

Typical impurities that need to be removed after the capture step include the residual host cell proteins and nucleic acids that were not entirely eliminated during the capture step, potential affinity ligands that leached from the chromatographic medium, as well as fragments, aggregates, and variants of the protein of interest [20]. The latter correspond to proteins that vary slightly in their primary sequence as compared to the target protein. These variations are typically the result of posttranslational modifications, for example due to protein oxidation, deamidation, or different glycosylations. Protein variants are usually difficult to remove due to their high similarity with the target protein.

Ion-exchange chromatography (IEC) or hydrophobic interaction chromatography (HIC) are typically employed during the polishing steps [22], exploiting differences in charge and hydrophobicity, respectively, between the solutes to be separated [21]. The use of size exclusion chromatography (SEC) has also been reported for the large-scale purification of antibodies and notably for the removal of aggregates [22]. The fundamental principles underlying the separation mechanisms by chromatography will be described in Chapter 2, while the multicolumn processes relevant for the polishing steps will be presented in Chapter 5.

As for the capture step, nonchromatographic techniques can also be envisaged for the polishing steps. For instance, the purification process of recombinant insulin involves the precipitation of impurities by ethanol addition followed by insulin crystallization [39].

1.2.5 Viral Clearance

As mentioned earlier, the removal of viruses is a major concern for the biopharmaceutical industry, and conventional purification processes typically target a reduction of the level of retroviruses by 12–18 orders of magnitude [40]. Several methods are available for *viral clearance*, such as low-pH inactivation, heat inactivation, filtration, solvents/detergents treatment, and gamma irradiation [40, 41]. Chromatographic steps have also been shown to reduce the content of viruses by several orders of magnitude [40].

The European Medicines Agency recommends "to investigate the contribution of more than one production step for virus reduction and at least two orthogonal steps should be assessed" [42]. For mAbs, a low-pH hold after the capture step and a viral filtration operation after the polishing steps are typically sufficient to meet the

specifications in terms of virus clearance [22]. Specific process development is needed to extend these methods to continuous manufacturing [43].

1.2.6 Formulation

After the purification process, a *formulation step* is necessary to obtain the therapeutic protein at the desired concentration and in the selected formulation solution, which usually contains a buffering agent to regulate the pH as well as various excipients (e.g., salts, polyols, amino acids, surfactants). A proper formulation solution is essential to ensure proper drug delivery into the organism and to guarantee a sufficiently long shelf life of the biopharmaceutical product [44–47]. With this respect, the strong tendency of proteins to form aggregates represents a major issue because the presence of aggregates may compromise drug efficacy and drug safety. The impact of formulation conditions on protein stability will be discussed in Chapter 7.

Diafiltration (DF) and ultrafiltration (UF) are commonly used to perform buffer exchange and to increase protein concentration, respectively [22, 48, 49]. UF and DF processes are typically performed with the tangential flow filtration (TFF) technology, where the feed stream flows parallel to the filter surface in order to prevent fouling or clogging of the system [48, 49].

Finally, the manufacturing process ends with a sterile filtration step and fill/finish operations, which are sometimes performed by specialized contractors [22, 50].

To date, the development of antibody-based drugs has focused primarily on injectable routes of administration, thus favoring the choice of liquid formulations. These are in general cheaper and faster to develop than alternative formulation types. However, proteins in solution are prone to physical and chemical modifications (e.g., unfolding, aggregation, fragmentation, oxidation, deamidation), which prompted the use of lyophilized formulations in several cases including some blood factors, growth hormones, antibodies, and PEGylated interferon [44, 51, 52].

1.2.7 Additional steps

Protein Refolding

As mentioned in Section 1.2.1, high expression levels of proteins in bacteria often result in the formation of inclusion bodies containing the protein of interest in an inactive aggregated state. In this case, an additional step is required in the production process so as to solubilize and properly refold the therapeutic protein [15]. Protein refolding is performed by exchanging the buffer used to solubilize the protein with a buffer favoring native protein conformations. It has been shown that this step is facilitated by the use of chromatographic columns, although the underlying mechanisms of matrix-assisted refolding has not been fully elucidated yet [21, 53].

Protein Conjugation

Protein-based drugs have emerged as a major class of pharmaceuticals due to their outstanding targeting properties. However, therapeutic proteins often suffer from a rapid

in vivo clearance, a low solubility, and a limited stability. A possibility to overcome these limitations is to attach a suitable chemical molecule to the protein. The idea is to benefit from the targeting properties of the protein, while further enhancing its therapeutic action by a controlled chemical modification [54]. For example, the attachment of polyethylene glycol (PEG) chains to small proteins has been shown to improve the *in vivo* circulation half-life of the drug [55]. Moreover, the conjugation of antibodies targeting cancer cells to cytotoxic drugs, the so-called antibody drug conjugates (ADC), has led to significant improvements in the field of oncology [56]. Protein conjugation is thus highly important for the pharmaceutical industry, and Chapter 6 will be dedicated to this topic, addressing both reaction and purification aspects.

1.3 Overview of the Impurities to Be Removed

1.3.1 Process-Related and Product-Related Impurities

In Section 1.2, we have enumerated a number of impurities that need to be removed during the downstream processing of recombinant proteins. Figure 1.2 presents an overview of these impurities for a typical mAb production process. Following the nomenclature of the international council for harmonization (ICH), impurities may be classified as *product-related impurities* and *process-related impurities* [57].

Product-related impurities are similar to the target protein and include protein aggregates, protein fragments, and various protein variants such as glycoforms, deamidated proteins, and oxidized proteins. Among these impurities, protein aggregates are of

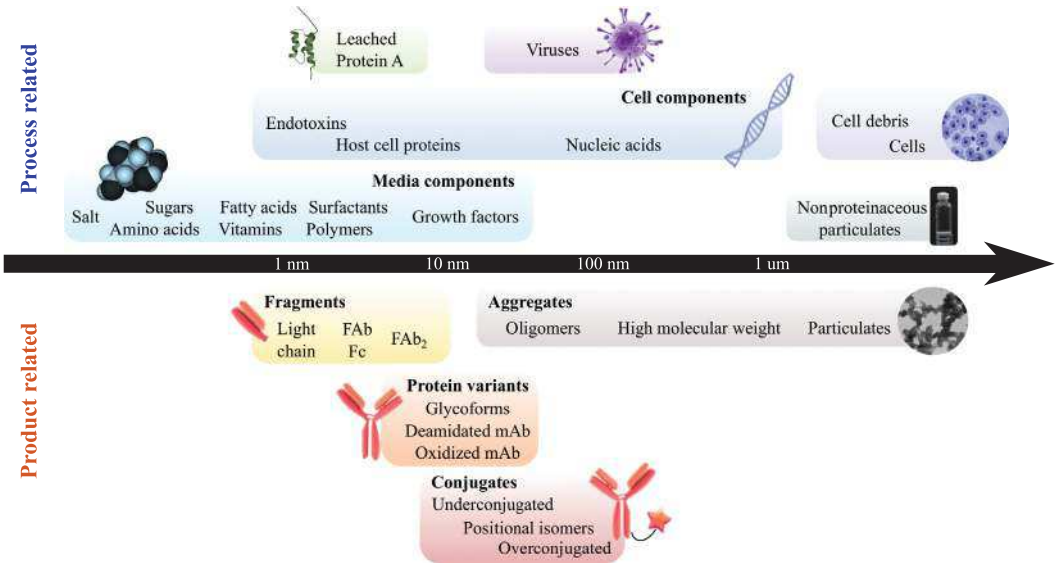

Figure 1.2 Summary of the main process-related impurities and product-related impurities encountered in the manufacturing of mAbs.

particular concern because they may affect drug efficacy and safety [58, 59]. The formation of protein aggregates and their removal are topics covered in Chapter 7.

When dealing with conjugated proteins, the situation is possibly more complex because the conjugation reaction typically leads to a mixture of conjugates, which differ in terms of both number and position of the grafted molecules. This implies that additional impurities need to be removed, such as unconverted proteins, residual chemical reactants, by-products of the reaction, and under-, over- and misconjugated proteins. Strategies to purify conjugated proteins will be addressed in Chapter 6.

On the other hand, process-related impurities do not share common features with the target protein. They derive from the manufacturing process and include host cell proteins, nucleic acids, viruses, and leached affinity ligands.

Overall, when considering all the aforementioned impurities, two main requirements of the purification process can be identified: *(i)* it should remove numerous and diverse impurities in a limited number of steps, *(ii)* it should separate molecules that differ only by slight variations in their size, charge, or hydrophobicity.

Chromatography is currently the technique of choice for the purification of biopharmaceuticals as it is a versatile, selective, and efficient separation technique satisfying these two points. Chromatography is extensively used to purify mAbs [20, 38], a wide range of other recombinant proteins [21, 60], nonrecombinant proteins (e.g., purification of plasma proteins) [25, 61, 62] as well as synthetic peptides [63].

1.3.2 Purity Specifications

The purity requirements for biopharmaceuticals are specific to each product as they depend on various criteria, such as the therapeutic indication or the dose administrated. With regard to viruses, ICH guidelines recommend to review viral clearance studies on a case-by-case basis according to a risk assessment analysis [64, 65]. There is also no general rule on the maximum limit for the aggregate content in biopharmaceuticals because the toxicity of aggregates may vary from one protein to another [66]. Nevertheless, in order to give a rough idea of the purity specifications of biopharmaceuticals, some indications for few relevant impurities are summarized in Table 1.1 [21].

As mentioned earlier, recombinant proteins are rarely produced as individual molecular entities, but rather as a large number of similar variants, also called isoforms, which vary by subtle differences only. Despite a growing understanding of the protein structure–function relationship, the impact of various isoforms on the biological and pharmaceutical activity of a drug remains largely unclear and can only be assessed

Table 1.1. Examples of indications for purity specifications.

Aggregates	<1.0%
HCP	<100 ng/mg
Ligand leakage	<1 ppm
Viruses	<1 particle per million doses
DNA	<10 pg per dose

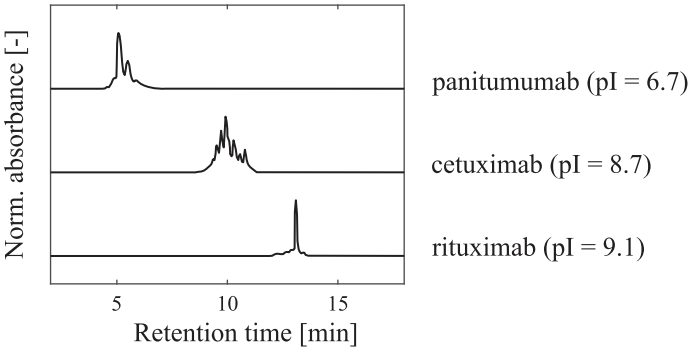

Figure 1.3 Chromatograms of three commercial mAbs obtained by analytical cation exchange chromatography using a pH gradient, illustrating the heterogeneity of biopharmaceutical products in terms of charge variants. The three selected mAbs elute at different times due differences in their isoelectric points (pI). Adapted from [67], copyright 2015, with permission from Elsevier.

by experimentation [16]. In order to guarantee drug quality, efficacy, and safety, it is paramount to maintain the distribution of these isoforms within established acceptable ranges in agreement with the ICH guidelines [57]: "An inherent degree of structural heterogeneity occurs in proteins due to the biosynthetic processes used by living organisms to produce them; therefore, the desired product can be a mixture of anticipated post-translationally modified forms (e.g., glycoforms). [. . .] The manufacturer should define the pattern of heterogeneity of the desired product and demonstrate consistency with that of the lots used in preclinical and clinical studies." For illustrative purposes, the degree of heterogeneity of some marketed biopharmaceutical products can be appreciated in Figure 1.3, which shows the chromatograms of three mAbs obtained by analytical cation exchange chromatography [67]. Each peak can be associated with at least one mAb variant.

Although it is challenging to control precisely the distribution of product variants, it is possible to influence the type and amount of these variants by acting both on the upstream and downstream parts of the production process. These considerations become particularly relevant in the case of biosimilars, where it is crucial to demonstrate that there are "no clinically meaningful differences" with respect to the reference product [68, 69].

1.4 Continuous Production Processes

In the previous sections, we described briefly the unit operations that are commonly used in the production of protein-based drugs. These considerations are sufficiently general to hold true for both batch and continuous manufacturing processes. The goal of this section is to compare these two modes of production, first in general terms and then with specific reference to biopharmaceutical processes.

1.4.1 Definition of Batch and Continuous Processes

A discussion on batch and continuous processes requires first to give clear definitions of these two terms, which is probably less obvious than one would expect. Generally, a process is termed *continuous* if it is constituted by open units with uninterrupted inlet and outlet material flows. *Batch processes* are instead constituted by units that are prevalently closed, and the feed materials/final products are loaded/withdrawn according to some time schedule. A domestic dishwasher represents a common example of a batch installation, where a given quantity of dishes is introduced in the device, undergoes successive operations (such as washing, rinsing, and drying) and is then taken out before another washing program is started. If the operations are repeated over regular periods (for example, the washing program is started every morning), the process is termed *periodic*. On the other hand, the continuous equivalent of the domestic dishwasher would be a conveyor washing machine used in large dining facilities. Importantly, while in the batch process, cleaning operations are performed one after the other in a single unit; they are performed simultaneously but at different locations along the conveyor belt in the continuous process, allowing constant inlet/outlet of dirty/clean dishes.

It is worth noting that the notion of constant inlet/outlet depends on the scale of observation and on the level of averaging. For example, the number of dishes cleaned per minute with the conveyor washing machine is constant during the working hours of the restaurant. However, if one looks at the number of dishes cleaned per minute over a week, one observes a discontinuous process, with peaks at lunch and dinner times. On the other hand, even though the overall cleaning process in a domestic dishwasher is discontinuous, single operations may be regarded as continuous at the time scale of the considered operation and for some material flows. This is, for example, the case for water (but of course not for the dishes) during the rinsing step if water is injected and evacuated at constant flow rates. It is thus clear that the distinction between "batch" and "continuous" processes is necessarily associated with a relevant time scale and an observed variable.

Moreover, it is important to avoid confusion between the concepts of being continuous, which as mentioned earlier characterizes a system with uninterrupted inlet and outlet flows, and being at *steady state*, where all the internal variables of the units (e.g., temperature, pressure, reactant concentration) are constant in time.

Considering the particular case of cell culture, several reactor types can be envisaged [19]. *Batch bioreactors* are rather rare because the depletion of nutrients eventually causes a decrease in cell viability, which is defined as the ratio of the number of viable cells over the total number of cells. Therefore, *fed-batch bioreactors*, where some feed solution is introduced intermittently, are usually preferred. On the other hand, two types of continuous bioreactors can be considered, namely *chemostats* and *perfusion bioreactors*. In both cases, the cells are introduced in the bioreactor before starting to operate the process, so that the continuous inlet flow contains only the culture medium and no cells. However, the two bioreactors differ regarding their outlet flows. Chemostats are characterized by a single continuous outlet flow containing the supernatant and the cells in the same proportions as inside the bioreactor. On the other hand, perfusion bioreactors