Chapter 1

Overview of bioseparations engineering

1.1. Introduction

Biotechnology has undergone phenomenal growth in recent years. Most of this has been in fundamental areas linked to cellular and molecular biology, biochemistry and biophysics. In comparison, growth in more applied areas such as *bioprocess engineering* has been relatively modest, largely due to significantly lower funding in these areas, both in academia and industry. The net result of this has been the hugely inflated but largely unfulfilled expectations about the benefits of biotechnology. It is now being appreciated that symmetric growth in basic and applied areas is crucial for healthy development of biotechnology. One of the major segments within biotechnology where research and development is vital is *bioprocessing* which deals with the manufacture of biochemicals, biopharmaceuticals, foods, nutraceuticals, and agrochemicals. A plethora of new biologically derived products have been developed, approved and licensed in the last decade. This includes monoclonal antibodies used for the treatment of cancer and multiple sclerosis, plasmids for gene therapy, cytokines and interleukins. Many of these products need to be extensively purified before they can be used for their respective applications. *Bioseparations engineering* refers to the systematic study of the scientific and engineering principles utilized for the large-scale purification of biological products. It is a broader term than the slightly dated *downstream processing* which specifically referred to the separation and purification segment of a bioprocess which followed some form of biological reaction e.g. purification of an antibiotic following microbial fermentation. However, the manufacture of several types of biological products does not involve *in-vitro* biological reactions. These products are synthesized *in vivo* in their respective

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natural sources and these are simply recovered using appropriate techniques e.g. manufacture of plasma proteins from blood, extraction of alkaloids from plants, extraction of enzymes from animal tissue. Bioprocessing can be broadly classified into two categories (see Fig. 1.1):

1. Reactive bioprocessing
2. Extractive bioprocessing

In reactive bioprocessing, the bioseparation process follows some form of biological reaction whereas extractive bioprocessing almost entirely involves bioseparation. In the context of reactive bioprocessing, upstream processing involves steps such as biocatalyst screening, enrichment, isolation and propagation, cell manipulation by recombinant DNA technology or hybridoma technology, media optimization and formulation, and so on. The biological reaction involved could be fermentation (i.e. cultivation of bacterial or fungal cells), cell culture (i.e. cultivation of animal or plant cells) or simply an enzymatic reaction. With extractive bioseparation, upstream processing involves raw material acquisition and pre-treatment.

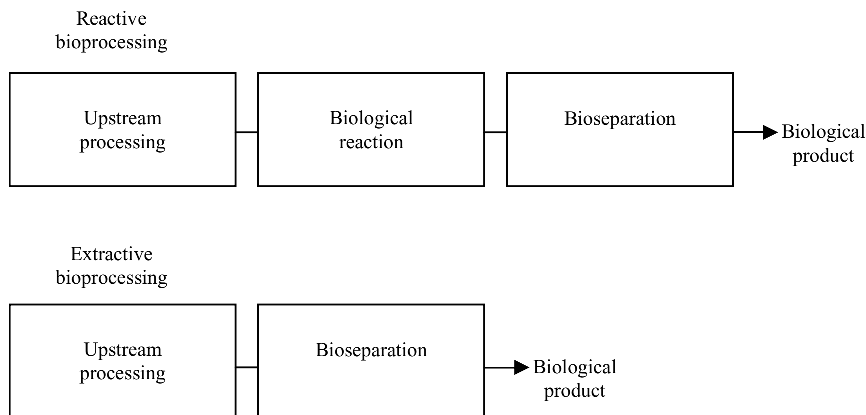

Fig. 1.1 Bioprocessing

1.2. What is separated in bioseparation?

Biologically derived products can be categorized in different ways, one way being based on their chemical nature (see Table 1.1).

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Table 1.1 Biological products classified based on chemical nature

Categories	Examples
Solvents	Ethanol, acetone, butanol
Organics acids	Citric acid, lactic acid, butyric acid
Vitamins	Ascorbic acid, vitamin B12
Amino acids	Lysine, phenylalanine, glycine
Antibiotics	Penicillins, rifanpicin, streptomycin, framycetin, gentamycin
Sugars and carbohydrates	Glucose, fructose, starch, dextran, xanthan, gellan
Lipids	Glycerol, fatty acids, corticosteroids, prostaglandins
Nucleic acids	Plasmids, therapeutic DNA, retroviral vectors, anti-sense oligonucleotides, ribozymes
Semi-purified proteins	Industrial enzymes, egg proteins, milk proteins, whey protein, soy proteins
Purified proteins	Therapeutic enzymes, monoclonal antibodies, plasma proteins, cytokines, interleukins, hormones, growth factors, diagnostic enzymes, vaccines
Cells	Bakers yeast, brewers yeast, freeze dried lactobacillus
Crude cellular extracts	Yeast extract, soy extracts, animal tissue extract
Hydrolysates	Soy hydrolysates, whey hydrolysates, animal tissue hydrolysates

Biological products can also be classified based on their intended applications (Table 1.2):

Table 1.2 Biological products classified based on application

Categories	Examples
Industrial chemicals	Solvents, organic acids, industrial enzymes
Agrochemicals	Biofertilizers, biopesticides
Biopharmaceuticals	Antibiotics, hormones, monoclonal antibodies, plasma proteins, vaccines, hormones, cytokines, therapeutic nucleic acids
Food and food additives	Whey proteins, milk proteins, egg proteins, soy proteins, protein hydrolysates
Nutraceuticals	Vitamins, enzymes, coenzymes, cofactors, amino acids, purified whey proteins
Diagnostic products	Glucose oxidase, peroxidase, HCG
Commodity chemicals	Detergent enzymes, insecticides
Laboratory reagents	Bovine serum albumin, ovalbumin, lysozyme
Cosmetic products	plant extracts, animal tissue extracts

1.3. Economic importance of bioseparation

The purification of biological products from their respective starting material e.g. cell culture media is technically difficult and expensive. This could frequently be the critical limiting factor in the

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commercialization of a biological product. In many cases bioseparation cost can be a substantial component of the total cost of bioprocessing. Table 1.3 summarizes the bioseparation cost of different categories of biological products. For proteins and nucleic acids, particularly those used as biopharmaceuticals, the bioseparation cost is quite substantial.

Table 1.3 Cost of bioseparation

Products	**Bioseparation cost (%)**
Solvents	15-20
Cells	20-25
Crude cellular extracts	20-25
Organics acids	30-40
Vitamins and amino acids	30-40
Gums and polymers	40-50
Antibiotics	20-60
Industrial enzymes	40-65
Non-recombinant therapeutic proteins	50-70
r-DNA products	60-80
Monoclonal antibodies	50-70
Nucleic acid based products	60-80
Plasma proteins	70-80

1.4. Nature of bioseparation

Bioseparation is largely based on chemical separation processes. A plethora of well established separation techniques is used in the chemical industry. A number of these techniques were found to be suitable for carrying out biological separations. However, while borrowing from chemical separations, the fundamental differences between synthetic chemicals and biological substances need to be kept in mind. Some biologically derived substance such as antibiotics and other low molecular weight compounds such as vitamins and amino acids are purified using conventional separation techniques such as liquid-liquid extraction, packed bed adsorption, evaporation and drying with practically no modifications being necessary. However, substantially modified separation techniques are required for purifying more complex molecules such as proteins, lipids, carbohydrates and nucleic acids. Often totally new types of separation techniques have to be devised. Some of the attributes of bioseparation which distinguish it from chemical separation are:

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1. Biological products are present in very low concentrations in the starting material from which they are purified. For example, monoclonal antibodies are typically present in concentrations around 0.1 mg/ml in the mammalian cell culture supernatants. Hence large volumes of dilute product streams have to be processed for obtaining even modest amounts of pure products.
2. Several other substances which are usually impurities and in some instances by-products are present in the starting material along with target biological products. Frequently these impurities or by-products have chemical and physical properties similar to those of the target product. This makes separation extremely challenging. Hence, bioseparation has to be very selective in nature.
3. There are stringent quality requirements for products used for prophylactic, diagnostic and therapeutic purposes both in terms of active product content as well as in terms of the absence of specific impurities. Injectable therapeutic products should be free from endotoxins and pyrogens. Solutions for such specific requirements have to be built into a bioseparation process.
4. Biological products are susceptible to denaturation and other forms of degradation. Therefore bioseparation techniques have to be "gentle" in terms of avoiding extremes of physicochemical conditions such as pH and ionic strengths, hydrodynamic conditions such as high shear rates, and exposure to gas-liquid interfaces. Organic solvents which are widely used in chemical separations have relatively limited usage in bioseparations on account of their tendency to promote degradation of many biological products.
5. Many biological products are thermolabile and hence many bioseparation techniques are usually carried out at sub-ambient temperatures.
6. Bioseparation is frequently based on multi-technique separation. This will be discussed in detail in a subsequent section.

1.5. Basis of separation in bioseparation processes

Biological products are separated based on one or more of the following factors:

1. Size: e.g. filtration, membrane separation, centrifugation

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2. Density: e.g. centrifugation, sedimentation, floatation
3. Diffusivity: e.g. membrane separation
4. Shape: e.g. centrifugation, filtration, sedimentation
5. Polarity: e.g. extraction, chromatography, adsorption
6. Solubility: e.g. extraction, precipitation, crystallization
7. Electrostatic charge: e.g. adsorption, membrane separation, electrophoresis
8. Volatility: e.g. distillation, membrane distillation, pervaporation

1.6. Physical forms separated in bioseparation

Bioseparation usually involves the separation of the following physical forms:

1.6.1. Particle-liquid separation

Examples of particle-liquid separation include the separation of cells from cell culture medium, the separation of blood cells from plasma in the manufacture of plasma proteins, and the removal of bacteria and viruses from protein solutions. Particle-liquid separation can be achieved by forcing the suspension through a porous medium which retains the particles while allowing the liquid to go through. This principle is utilized in filtration and membrane separation. Particle-liquid separation can also be achieved by subjecting the suspension to natural or artificially induced gravitational fields. If the particles are denser than the liquid medium in which they are suspended, these would settle and form a zone with very high particulate concentration. This is referred to as the sediment and the clear liquid left behind is referred to as the supernatant. This principle is utilized in separation processes such as sedimentation and centrifugation. If the particles are lighter than the liquid in which they are suspended, these would tend to float and hence concentrate near the top of the container in which the suspension is held. This principle is utilized in floatation.

1.6.2. Particle-particle separation in liquid medium

Examples of particle-particle separation in liquid medium include the fractionation of sub-cellular organelle, the separation of plasmid DNA from chromosomal DNA, and the separation of mature cells from young cells. This type of separation can be achieved by zonal centrifugation which involves the introduction of the mixture at a location within a

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liquid medium which is then subjected to an artificially induced gravitational field. As a result of this the heavier particles would migrate faster than the lighter particles, resulting in their segregation into distinct bands from which these particles can be subsequently recovered using appropriate means. Particle-particle separation can in theory be carried out by using a porous medium which retains the bigger particles but allows the smaller particles to go through. However, this sounds easier than it actually is and can only be carried out if the larger particles can be prevented from blocking the porous medium.

1.6.3. Particle-solute separation in liquid medium

An example of this is the separation of dissolved antibiotics from cells and cell debris present in fermentation broth. The methods used for particle-solute separation are fundamentally similar to those used for solid-liquid separation on account of the fact that the solute remains dissolved in the liquid medium.

1.6.4. Solute-solvent separation

Solute-solvent separation is quite common in bioseparation, the purpose of this being either the total or partial removal of a solvent from a solute product (e.g. protein concentration enrichment), or the removal of dissolved impurities from a liquid product, or the replacement of a solvent from a solution by another (i.e. solvent exchange). A range of options are available for solute-solvent separation the easiest of these being evaporation and distillation. However, these techniques involve the application of heat and cannot therefore be used for separation of biological materials which tend to be thermolabile. Membranes which can retain dissolved material while allowing solvents through are widely used for this type of separation: a reverse osmosis membrane will retain small molecules and ions, a nanofiltration membrane will retain larger molecules such as vitamins, hormones and antibiotics, while an ultrafiltration membrane will retain macromolecules such as proteins and nucleic acids. Another way of removing a solvent from a solute is by reversibly binding the solute on to a solid surface, this being referred to as adsorption. Once solute binding has taken place, this separation is transformed to a particle-liquid separation, i.e. the solvent is separated from the solid-bound solute. The bound solute is subsequently recovered from the solid material, this being referred to as desorption. An indirect method for solute-solvent separation is by inducing precipitation of the

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solute, thereby once again transforming the separation to a particle-liquid separation. Solvent exchange can also be carried out by liquid-liquid extraction where the solute is transferred from a liquid to another with which the original solvent is immiscible.

1.6.5. Solute-solute separation in liquid medium

Solute-solute separation is by far the most challenging form of separation. An example of this is the separation of serum albumin from other serum proteins. Solute-solute separation can be achieved by selective adsorption, i.e. by selectively and reversibly binding the target solute on to a solid material. Solute-solute separation can also be carried out by liquid-liquid extraction, i.e. by contacting the solution with an immiscible liquid in which the target solute has high solubility. With the advent of membranes, solute-solute separation has become a lot easier. Nanofiltration, ultrafiltration and dialysis membranes can be used for such separations. An indirect way of carrying out solute-solute separation is by precipitation, which involves the selective precipitation of the target solute. This separation is then transformed to that of particle-solute separation in liquid medium.

1.6.6. Liquid-liquid separation

Liquid-liquid separation is required in the manufacture of solvents such as acetone and ethanol which typically have to be separated from an aqueous medium. If the solvent is immiscible with water, phase separation followed by decantation may be sufficient. However, if the solvent is miscible with water (as in the case of ethanol), other separation methods have to be utilized. With temperature stable and volatile solvents such as ethanol, distillation has been traditionally used. However with the advent of membrane technology, separation processes such as membrane distillation and pervaporation have come into widespread use.

1.7. Bioseparation techniques

A plethora of bioseparation techniques is now available. Table 1.4 categorizes bioseparation techniques into two broad groups. As previously mentioned, a bioseparation process must combine high selectivity (or resolution) with high throughput (or productivity). Quite clearly none of those listed in the table can deliver this on their own.

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Hence bioseparation processes tend to be based on multiple techniques arranged such that both high-resolution and high-throughput can be obtained in an overall sense.

Table 1.4 Bioseparation techniques

Low-resolution + high-throughput	**High-resolution + low-throughput**
Cell disruption	Ultracentrifugation
Precipitation	Chromatography
Centrifugation	Affinity separation
Liquid-liquid extraction	Electrophoresis
Leaching	
Filtration	
Supercritical fluid extraction	
Microfiltration	
Ultrafiltration	
Adsorption	

1.8. The RIPP scheme

While developing a bioseparation process the following should be taken into consideration:

1. The nature of starting material: e.g. a cell suspension, a crude protein solution
2. The initial location of the target product: e.g. intracellular, extracellular, embedded in solid material such as inclusion bodies
3. The volume or flow-rate of the starting material
4. The relative abundance of the product in the starting material, i.e. its concentration relative to impurities
5. The susceptibility to degradation e.g. its pH stability, sensitivity to high shear rates or exposure to organic solvents
6. The desired physical form of the final product, e.g. lyophilized powder, sterile solution, suspension
7. The quality requirements, e.g. percentage purity, absence of endotoxins or aggregates
8. Process costing and economics

A RIPP (Recovery, Isolation, Purification and Polishing) scheme is commonly used in bioseparation. This strategy involves use of low-resolution techniques (e.g. precipitation, filtration, centrifugation, and crystallization) first for recovery and isolation followed by high-resolution techniques (e.g. affinity separations, chromatography, and

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electrophoresis) for purification and polishing. The high-throughput, low-resolution techniques are first used to significantly reduce the volume and overall concentration of the material being processed. The partially purified products are then further processed by high-resolution low-throughput techniques to obtain pure and polished finished products.

1.9. Example of bioseparation

A scheme for the bioseparation of reagent grade monoclonal antibody from cell culture supernatant is shown in Fig. 1.2. Murine or mouse monoclonal antibodies are produced by culturing hybridoma cells in different types of bioreactors. In recent years it has been possible to synthesize humanized and chimaeric monoclonal antibodies by culturing recombinant Chinese Hamster Ovarian (CHO) cells. In the bioseparation scheme shown in Fig. 1.2, the key purification step involves affinity chromatography. Prior to affinity chromatography the cell culture supernatant needs to be cleaned up by membrane filtration or centrifugation so that cells, cell debris and other particulate matter do not clog-up the affinity column. The nearly purified monoclonal antibody obtained by affinity chromatography is further purified by ion-exchange chromatography and polished by gel-filtration to obtain greater than 98% pure product in the solution form. This percentage purity figure is relative to other proteins present in the product. The antibody solution is then filtered to remove bacterial contaminant and marketed either as a sterile solution or as a freeze dried powder. The scheme for purifying therapeutic grade monoclonal antibodies would be largely similar to that shown in Fig. 1.2. In addition to the basic purification scheme used for making the reagent grade monoclonal antibody, some additional steps for removing particulate matter and specific impurities such as endotoxins and antibody dimers and higher order aggregates would be required. An additional step to formulate the monoclonal antibody in an appropriate buffer would also be required.

1.10. Current trends in the bioseparation

The main disadvantages of using the RIPP scheme are:

1. High capital cost
2. High operations cost
3. Lower recovery of product

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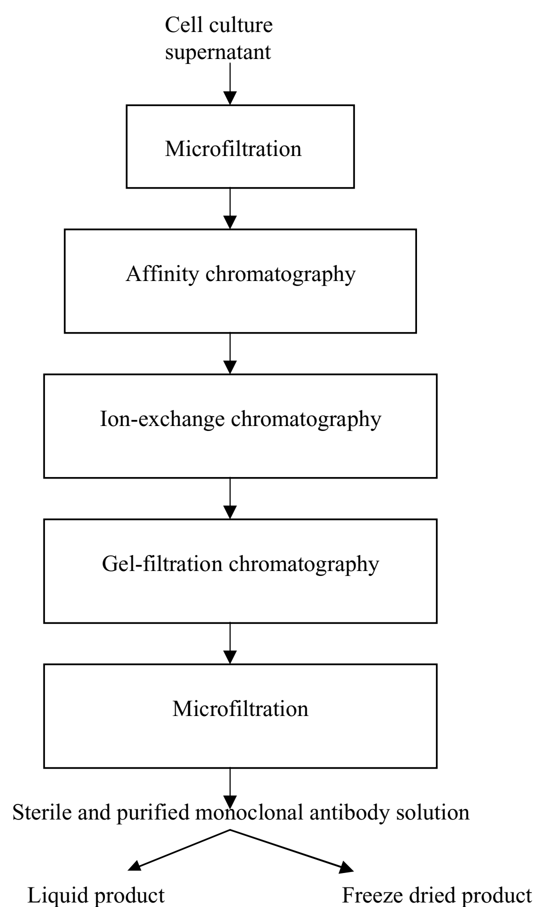

Fig. 1.2 Purification of reagent grade monoclonal antibody

With the advent of membrane separation processes and other new types of separations, the potential exists for avoiding the conventional RIPP scheme. Membrane processes give high throughput and can be fine-tuned or optimized to give very high selectivity. The use of these new techniques can significantly cut down the number of steps needed for bioseparation. Some of these new and emerging techniques are:

1. Membrane and monolith chromatography
2. Expanded-bed chromatography
3. High-resolution ultrafiltration
4. Hybrid bioseparations

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