Separation Techniques/Bioseparation

Ph.D. Courses

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Reference book: "Bioseparations: Principles and Techniques" by Sivasankar

This course introduces the fundamental principles of separation operations for the recovery of products from biological processes, membrane filtration, centrifugation, cell disruption, extraction, and process design.

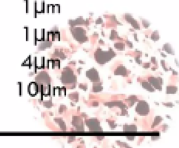
Bioseparation is the name given to the practice of purifying biological products on a large scale, using fundamental aspects of engineering and scientific principles. The end goal of bioseparation is to refine molecules, cells, and parts of cells into purified fractions. Biological products can be separated and purified depending on the following characteristics: density, diffusivity, electrostatic charge, polarity, shape, size, solubility, and volatility.

Bioproducts Chemical substances or combinations of chemical substances that are made by living things. Bioproducts can be broadly classified into three categories of sources:

- Whole cells: single-cell protein, baker's yeast, and animal feed supplements derived from yeast fermentation.
- Intracellular macromolecules: protein in inclusion bodies(nuclear, cytoplasmic, or periplasmic aggregates of bio-macromolecules, mostly proteins) from recombinant bacterial fermentations, starch in inclusion bodies found in plant cells and intracellular proteins.
- Extracellular products: proteins, antibiotics, organic acids, and alcohols secreted during microbial fermentations or cell culture

Categories of Bioproducts and Their Sizes

Bioproduct	Examples	Molecular Weight (Da)	Typical radius
Small molecules	Sugars Amino acids Vitamins Organic acids	200-600 60-200 300-600 30-300	0.5nm 0.5nm 1-2nm 0.5nm
Large molecules	Proteins Polysaccharides Nucleic acids	10^3-10^6 10^4-10^7 10^3-10^{10}	3-10nm 4-20nm 2-1,000nm
Particles	Ribosomes Viruses Bacteria Organelles Yeast cells Animal Cells		25nm 100nm 1µm 1µm 4µm 10µm



Differences Between Bioseparation and Chemical Separation

Although bioseparation is based on traditional chemical separation processes, they do differ in significant ways. This is because the materials being purified and separated in bioseparation are biological substances rather than the synthetic chemicals used in traditional techniques. As such, substances such as proteins, carbohydrates, and nucleic acids are not suitable for the rigors of traditional techniques like packed-bed adsorption and evaporation.

Often, the desired final product is only found in very minute quantities in the starting substance from which they are refined. Because of this, vast quantities of dilute product streams must undergo processing in order to obtain a small amount of pure product. Meanwhile, there are often unwanted impurities in the starting substance that have a similar genetic makeup to the desired product, thus making separation very difficult.

Because biological products are more apt to degradation than chemical ones, this rules out the use of many common organic solvents in bioseparation, since they have a tendency to act as a catalyst for degradation. Furthermore, many biological substances are unstable when heated and as such have to be handled in sub-ambient temperatures.

Bioseparation Techniques

There are many different techniques by which bioseparation can be achieved – however, there are none that currently work effectively on their own. This is because bioseparation requires a combination of high resolution (also known as selectivity) with high throughput (also called productivity). As you will notice in the table of techniques below, not a single one combines those two traits.

As a result, bioseparation must incorporate two or more techniques to achieve dual proficiency in the two categories.

High Throughput & Low-Resolution	High Resolution & Low Throughput
Adsorption	Affinity Separation
Centrifugation	Chromatography
Filtration / Microfiltration / Ultrafiltration	Counter-current extraction
Precipitation	Electrophoresis
Solvent extraction	Ultracentrifugation
Supercritical fluid extraction	

One of the more commonly used methods of achieving bioseparation is through the deployment of a RIPP scheme (Recovery, Isolation, Purification, Polishing). This technique will first utilize one of the low-resolution methods from the left column above to achieve recovery and isolation of the desired product. Then, one of the high-resolution methods from the right column will purify the product and "polish" it. Polishing can refer

to sterilization, removal of contaminants, and any other final processing steps before it is packaged into a marketable form.

Classification of bioseparation steps (RIPP scheme)

Although a variety of bioseparation procedures exist, they can be classified into four distinct steps that include

- **Removal of insoluble (Solids):** Filtration, centrifugation, microfiltration
- **Isolation of product (volume reduction)** Cell disruption, extraction, adsorption, ultrafiltration, precipitation
- **Purification:** Adsorption, elution chromatography, ultrafiltration, electrophoresis, precipitation, crystallization
- **Polishing.** Crystallization, drying, auxiliary process, solvent recovery, water preparation

As is to be expected, a wide variety of bioseparation procedures are available. Because these processes contribute significantly to the cost of the product, the economic consequences of these processes must be carefully considered. the bioseparation processes include, but are not limited to, cell disruption, centrifugation, chromatography, drying, evaporation, extraction, filtration, membrane separation, and precipitation. Some of these processes are classical and their mechanisms of action are well documented in the literature. Some of the preceding processes still have to be proved, especially on the large-scale level.

The end product of interest to be obtained from these processes must meet varying, rather strict demands before it can be placed on the marketplace. For example, the product must be sterile; attain stringent quality requirements; and be free from detergents, endotoxins, proteases, etc. A pure product should satisfy the demands of

- **no immunogenic substances present**
- **no unwanted biological activity present**
- **no microbiological contamination**
- **no enzymatic activity present that is harmful to the product.**

For example, other proteins, modified proteins, nucleic acids, oligonucleotides, or nucleotides contribute to an immunogenic response. Enterotoxins and nonspecific activity (such as complement activation) contribute to unwanted biological activity.

In general, the end product quality requirements are largely dependent on the end use of the product. For therapeutic usage some of the requirements that are to be met include potency, identity, abnormal toxicity, nucleic acids, homogeneity, etc. The bioseparation process or protocol that is utilized to separate the product must satisfy these requirements at the end. Thus bioseparation processes are defined by the nature of the product and its application. In some cases, a high degree of purity is required, whereas in others simply the absence of conflicting activity is sufficient. During the initial bioseparation steps one attempts to maximize product yield even at the expense of retaining contaminants. These contaminants may be removed later using high-resolution fractionation processes. Besides, one has to be careful in the bioseparation protocol to maintain adequate containment of any potentially hazardous by-products.

One will require a wide variety of steps in the bioseparation protocol to meet different demands on the quality of the end product. However, one has to limit the number of steps; and one should get the most out of each step. Ideally, one should, if it is at all possible, try to restrict the bioseparation protocol to just two or three steps. One should attempt to obtain at least 90% of the product from each step. Thus, if we have two steps then the overall efficiency is 81%. If three steps are utilized, then the efficiency drops to about 73%. Note that three steps of efficiency of 80% each will eventually yield an overall efficiency of

51.2%. Thus, the need is to use as few steps as possible, and also to get as much as you can from each step.

Physical forms separated in bioseparation

1-Particle-liquid separation: the separation of cells from cell culture medium

2-Particle-particle separation in liquid medium: the separation of plasmid DNA from chromosomal DNA

3-Particle-solute separation in liquid medium: the separation of dissolved antibiotics from cells

4-Solute-solute separation in liquid medium: the separation of serum albumin from other serum proteins.

5-Liquid-liquid separation: the separation of solvents such as acetone and ethanol from an aqueous medium (?).

Selection of separation sequence

In the selection of separation sequence, five main rules provide a good basis for process selection which are as follows:

- **Rule 1:** Choose separation processes based on different physical, chemical, or biochemical properties.
- **Rule 2:** Separate the most plentiful impurities first.
- **Rule 3:** Choose those processes that will exploit the differences in the physicochemical properties of the product and impurities in the most efficient manner.
- **Rule 4:** Use a high-resolution step as soon as possible.
- **Rule 5:** Do the most difficult step last

Primary metabolites

Primary metabolites are involved in the growth, development, and reproduction of the organism. The primary metabolite is typically a key component in maintaining normal physiological processes; thus, it is often referred to as a central metabolite. Primary metabolites are typically formed during the growth phase as a result of energy metabolism and are deemed essential for proper growth. Examples of primary metabolites include alcohols such as ethanol, lactic acid, and certain amino acids. Within the field of industrial microbiology, alcohol is one of the most common primary metabolites used for large-scale production. Specifically, alcohol is used for processes involving fermentation which produce products like beer and wine. Additionally, primary metabolites such as amino acids– including L-glutamate and L-lysine, which are commonly used as supplements– are isolated via the mass production of a specific bacterial species, Corynebacteria glutamicum. Another example of a primary metabolite commonly used in industrial microbiology is citric acid. Citric acid, produced by Aspergillus niger, is one of the most widely used ingredients in food production. It is commonly used in pharmaceutical and cosmetic industries as well.

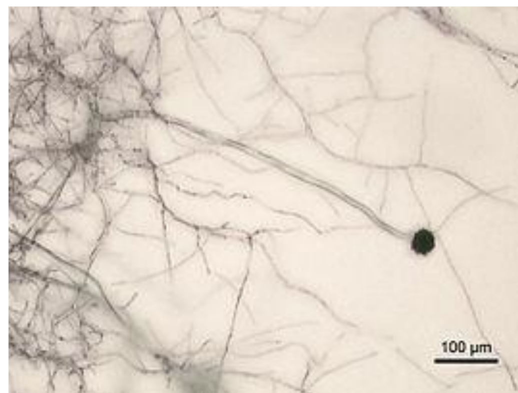


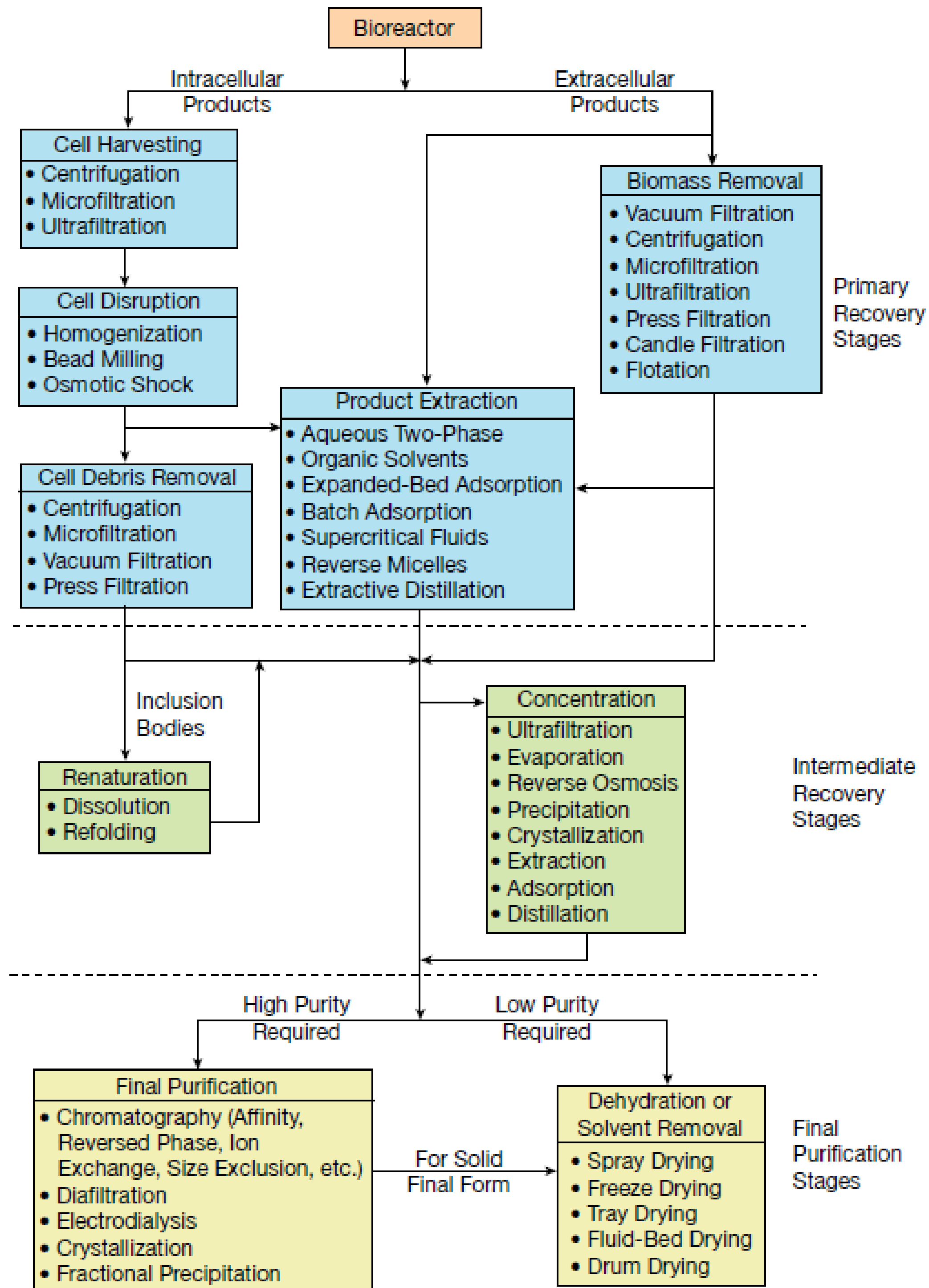
Figure: Aspergillus niger**: Microorganisms such as Aspergillus niger are used in industrial microbiology for mass production of citric acid.**

Secondary Metabolites

Secondary metabolites are typically organic compounds produced through the modification of primary metabolite synthases. Secondary metabolites do not play a role in growth, development, and reproduction like primary metabolites do, and are typically formed during the end or near the stationary phase of growth. Many of the identified secondary metabolites have a role in ecological function, including defense mechanism(s), by serving as antibiotics and by producing pigments. Examples of secondary metabolites with importance in industrial microbiology include atropine and antibiotics such as erythromycin and bacitracin. Atropine, derived from various plants, is a secondary metabolite with important use in the clinic. Atropine is a competitive antagonist for acetycholine receptors, specifically those of the muscarinic type, which can be used in the treatment of bradycardia. Antibiotics such as erythromcyin and bacitracin are also considered to be secondary metabolites. Erythromycin, derived from Saccharopolyspora erythraea, is a commonly used antibiotic with a wide antimicrobial spectrum. It is mass produced and commonly administered orally. Lastly, another example of an antibiotic which is classified as a secondary metabolite is bacitracin. Bacitracin, derived from organisms classified under Bacillus subtilis, is an antibiotic commonly used a topical drug. Bacitracin is synthesized in nature as a nonribosomal peptide synthetase that can synthesize peptides; however, it is used in the clinic as an antibiotic.

Figure: **Erythromycin tablets**: Erythromycin is an example of a secondary metabolite used as an antibiotic and mass-produced within industrial microbiology.

Development of a sequence of bioseparation



▲ **Figure 1.** A bioproduct recovery process typically involves some combination of these steps and operations. Source: Adapted from *(6)*.

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, and 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, and suspension.
7. The quality requirements, e.g. percentage purity, absence of endotoxins or aggregates.
8. Process costing and economics.

- **Challenges in bioseparation engineering**
- Low product concentration concentrations
- Large number of impurities,
- Thermolabile bioproducts.
- Narrow operating pH and ionic strength window
- Shear sensitivity of bioproducts
- Low solubility of bioproducts in organic solvents
- Instability of bioproducts in organic solvents
- Stringent quality requirements
- Percentage purity