SEPARATION PROCESSES IN BIOTECHNOLOGY

edited by

Juan A. Asenjo

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Separation Processes in Biotechnology

Bioprocess Technology

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Series Introduction

The revolutionary developments in recombinant DNA and hybridoma technologies that began in the mid-1970s have helped to spawn several hundred new business enterprises. Not all these companies are aimed at producing gene products or cell products, as such. Many are supportive in nature: that is, they provide contract research, processing equipment, and various other services in support of companies that actually produce cell products. With time, some small companies will probably drop out or be absorbed by larger, more established firms. Others will mature and manufacture their own product lines. As this evolution takes place, an explosive synergism among the various industries and the universities will result in the conversion of laboratory science into industrial processing. Such a movement, necessarily profit driven, will result in many benefits to humanity.

New bioprocessing techniques will be developed and more conventional ones will be revised because of the influence of the new biotechnology. As bioprocess technology evolves, there will be a need to provide substantive documentation of the developments for those who follow the field. It is expected that the technologies will continue to develop rapidly, just as the life sciences have developed rapidly over the past 10–15 years. No single book could cover all of these developments adequately. Indeed, any single book will be in need of replacement or revision every few years. Therefore, our continuing series in this rapidly moving field will document the growth of bioprocess technology as it happens.

The numerous cell products already in the marketplace, and the others expected to arrive, in most cases come from three types of bioreactors: (a) classical fermentation; (b) cell culture technology; and (c) enzyme bioreactors. Common to the production of all cell products or cell product analogs will be bioprocess control, downstream processing (recovery and purification), and bioproduct finishing and formulation. These major branches of bioprocess technology will be represented by cornerstone books, even though they may not appear first. Other subbranches will appear, and over time, the bioprocess technology "tree" will take shape and continue growing by natural selection.

W. Courtney McGregor

Foreword

The successful reduction-to-industrial-practice of modern biotechnology is the ultimate responsibility of the chemical and biochemical engineering profession. The explosive growth of scientific and commercial activity in the fields of basic and applied life sciences over the past decade has confronted the practicing chemical/biochemical engineer with the formidable problem of trying to keep abreast of developments at the life science laboratory frontier. Undoubtedly these developments will have an impact on industrial bioprocessing techniques and methods. Proliferation of the life science and biotechnology literature has rendered it virtually impossible for even the most meticulous practitioner to become (and remain) aware of advances in bioprocessing technology that are of essential importance to the manufacture of biological products. This book represents one of the first comprehensive efforts to deal with this critical problem.

University education in chemical engineering, at both the graduate and undergraduate levels, if it is to be responsive to the growing need for engineering expertise in industrial biotechnology, must structure its teaching and research programs to provide insight into the application of chemical engineering principles to the solution of applied life science problems. Very few academic institutions around the world today possess faculties with appropriate training and experience to organize and institute such programs, or to teach them effectively. There is, however, a consensus among chemical/biochemical engineering educators that proper preparation for a career in biotechnology requires awareness by the student that the basic, classical tools of the chemical engineer are essential to his capacity to deal competently with bioprocess engineering problems, and that developing confidence in applying these concepts to real bioprocess-

ing operations is central to proper training in this discipline. This book, with its excellent integration of life science fundamentals, chemical engineering principles, and the needs and practices of downstream bioprocessing, will be particularly useful to educators and students of biotechnology. Of particular importance to both practicing chemical engineers and chemical engineering educators will be the attention paid to modern chemical engineering concepts and their application to bioprocessing: namely, rigorous modeling, process simulation, process synthesis and optimization, large-scale equipment design, and process economics.

Increasing numbers of life scientists—particularly those involved in the biotechnology industry—are beginning to appreciate the importance of an understanding of the problems and limitations of bioproduct separation and purification in the selection and optimization of cells and cell-culture methods for the industrial production of biologicals. The critical role of cross-disciplinary interaction between life scientists and engineers early in the development of biotechnology processes is now widely acknowledged by industrial research and development managers in this field. Thus, there is considerable pressure building in both industry and academia to provide opportunities for basic life scientists to familiarize themselves with bioprocess engineering fundamentals. This book should serve a useful function in providing a broad overview of bioprocess principles and techniques for life science researchers interested in successful commercialization of the products of their creative efforts.

Dr. Asenjo is to be commended for having enlisted the participation of a distinguished group of internationally recognized experts to contribute to this landmark volume, and for having organized its contents in a rational and readable form. It will serve as a valuable reference and teaching text for many years to come.

Alan S. Michaels
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Preface

It is accepted today that advances in recovery, separation, and purification techniques used in bioprocesses will play a crucial role in the development of large-scale biotechnology. They are the aspects of bioprocess engineering most in need of attention, in particular for the production of novel products and proteins. Downstream operations are crucial stages in terms of maintaining product characteristics and activity aiming at high yield and purity.

The aim of this book is to be not just a collection of papers or monographs but an attempt to include in a comprehensive text the recovery, separation, and purification operations of bioprocess technology. To achieve this the editorial role has been an active one in order to avoid duplication and favor comprehensiveness. Individual chapters emphasize concepts that are, and will be, important in the development of large-scale versions of techniques that have been well established at the laboratory level. These concepts lay in the interface of basic physical biochemistry and biochemical engineering. This book should encourage the development of new ideas emerging from this interface between biological scientists and chemical engineers. This volume will be useful to basic biological scientists with an interest in scale-up, to people doing frontier research in bioprocess separations, to engineers interested in biological processes, and to those having to design actual downstream separation sequences and flowsheets in biotechnology, in both industry and academia. It should also serve as a textbook for graduate students taking advanced courses or carrying out research in the field.

Individual chapters are comprehensive and contain a balanced blend of theory, present state of the art, and important and recent practical applications. At the end of many chapters some problems

or exercises which are either numerical or of the essay type have been included. These will help in clarifying basic concepts and understanding in order to induce the development of new ideas necessary to build this emerging area.

This book has been divided into five parts according to the role of the separation operation in the downstream process. The first part describes fundamental features of physical, chemical, and biochemical characterization of the product and its contaminants, which is the first consideration to take into account when deciding on a downstream processing strategy. It also considers some questions on rationalizing the separation sequence by choosing a minimum number of necessary separation operations. The question of optimization and process synthesis is covered in more detail in the last part.

Part II concerns the extraction and release of products from the microbial cells. This is of main concern for heterologous proteins that microbial cells cannot secrete. It covers more traditional nonspecific operations such as homogenization and bead mill disruption and recent advances in protein secretion systems, differential product release, and membrane permeabilization by enzymatic and chemical methods which offer the attractive advantage of selectivity.

Part III covers main separation and concentration operations including ion-exchange processes used for this purpose, membrane processes, aqueous two-phase extractions, and precipitation. High resolution ion-exchange chromatography used for purification is covered in the following section. Filtration and centrifugation, and liquid-liquid extraction using organic solvents are not discussed in this section, but have been widely described in the literature and are fully reviewed in Chapter 19 on processing plants and equipment.

Part IV is on purification operations and has five chapters. Of the available large-scale protein purification operations, ion-exchange is probably by far the most widely used one. Affinity chromatography, on the other hand, represents the protein purification operation that most probably has the largest potential for very specialized proteins as it can be extremely specific and a very high purification factor can be obtained in one step. Reversed-phase chromatography (RPC) is largely used for assaying and preparing peptides, always in the "high pressure" mode (HPLC). Gel filtration is not specifically reviewed in this section but this operation is widely covered in the literature (Scopes, 1987; Wiseman, 1986) and is also documented in Chapter 19. In addition, gel filtration is not particularly suitable as a large-scale purification operation and is efficient only as an intermediate desalting operation or for final polishing (Pharmacia, 1983; Asenjo and Patrick, 1990).

Part V covers the more relevant process engineering aspects of downstream processing (DSP). It begins with a review of recent developments in process synthesis and design, stressing their potential for biotechnology. It includes an evaluation of rigorous computer methods in chemical engineering, recent developments in artificial intelligence (expert systems), and present trends in this expanding field. Recent developments of automation in chromatography (expert systems for control) are covered in Chapter 18. Chapter 19 is a thorough review of virtually all equipment used in DSP in biotechnology, not only in the more recent field of protein purification but also in the manufacture of pharmaceuticals, organic acids, and others. This very comprehensive chapter provides important information for process design and equipment selection. The final chapter is on economics. It has two sections. It first discusses general aspects of the relationship between economic viability and scale in biotechnology processes and then gives a detailed example of the economic evaluation of a typical modern biotechnology process.

Juan A. Asenjo

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Separation Processes in Biotechnology

I

SEPARATION PROCESSES

1

Selection of Operations in Separation Processes

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Separation and purification of a protein from a large-scale fermentation or cell rupture supernatant is a critical element of modern process biotechnology. It represents the major manufacturing cost, and therefore competitive advantage in production will depend not only on innovations in molecular biology, immunology, and other areas of basic biological sciences, but also on innovation and optimization of separation and downstream processes (Wheelwright, 1987).

The design of a large-scale process to economically purify a protein, maintaining a high yield, yet obtaining a virtually pure product while also minimizing the cost, requires three main considerations: (1) clearly defining the final product objective, (2) characterizing the starting material, and with these two pieces of information in hand (3) defining possible separation steps and constraints regarding the protein product, operations, and conditions to be used (Asenjo, 1988). The aim of this chapter is to analyze and discuss the main criteria used to select separation and purification steps on a rational basis.

I. DEFINING FINAL PRODUCT

At this stage it is necessary to have information on or (if this is not the case) to be able to define the final product to be obtained and its uses. What is the product going to be and how is it going to be used (if therapeutic, for humans or animals, how large doses, how many, how often)? Questions regarding the purity required are vital (e.g., 99%, 99.9%, or 99.98%), as well as allowable ranges of impurity concentrations. With therapeutic and recombinant proteins any impurities have to be minimized, whereas for the production of bulk indus-

trial enzymes this is not the case. For instance, in vaccine production it is necessary to remove all traces of unwanted immunogens to prevent potentially catastrophic immunological side reactions. Experience with fetal calf serum contamination of therapeutic proteins has given rise to a consensus that levels of contaminating protein of around 100 ppm should be acceptable from the product safety point of view (Cartwright, 1987). The major perceived hazard appears to be the persistence of nucleic acids with oncogenic potential. State-of-the-art assays (Cartwright, 1987) will allow detection of around 10 pg of nucleic acids, and it is generally agreed that final product contamination should not exceed 10 pg/dose. Common contaminants that will have side reactions even if present in very small amounts are pyrogens that mainly originate from bacterial cell walls. For these, in vivo testing using established methods is necessary; hence on-line control of such processes is very difficult to achieve.

II. CHARACTERIZATION OF STARTING MATERIAL

Probably the most crucial elements that will affect large-scale process design are the physical, chemical, and biochemical properties of the contaminating materials in the original broth and those of the protein that will constitute the final product. The properties of the starting material will be partly determined by its fermentation source, viz., bacterial, yeast, or mammalian cell, the type of cultivation medium used (e.g., presence of albumin, calf serum, proteases, solid bodies like whole cells or cell debris), the length of the fermentation, and whether the product is intracellular or extracellular. To these factors we must add the actual physicochemical properties of the product (surface charge/titration curve, biospecificity toward certain ligands, surface hydrophobicity, M.W., pI, stability) as compared to those of the contaminant components in the crude broth. The stability of the final product is also of utmost importance as this will affect the types of operations that can be used as well as the conditions and processing times that can be afforded.

III. SELECTION OF SEPARATION SEQUENCE

The next stage is to define realistic separation steps on the basis of all the information provided in Sections I and II. The following five main heuristics or rules of thumb, which are discussed in some detail in Chapter 17, provide a good basis for process selection:

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 arduous step last.

IV. PURIFICATION PROCESS AND UNIT OPERATIONS

The number of necessary steps in a large-scale protein purification procedure is usually not more than four or five and they can be divided into two main subprocesses, protein recovery and protein purification:

Protein recovery

1. Cell separation
2. Cell disruption and debris separation (for intracellular proteins only)
3. Concentration

Protein purification

4. Pretreatment or primary isolation
5. High-resolution purification
6. Polishing of final product

It is important to bear in mind that, as noted above, there will be interactions between the fermentation system and conditions and protein recovery; hence, production methods used will affect the later purification steps. Such interactions have been discussed in some detail (e.g., Wang, 1983; Fish and Lilly, 1984; Cartwright, 1987). It is important, then, to consider the process of fermentation and downstream processing as a single system so that, for example, the effect of decisions about the fermentation conditions on subsequent purification stages is made clear. Product concentration will partly depend on the reactor system used. The presence of nucleic acids and proteases as well as bacterial contamination must be minimized, which results in a need for rapid processing. The presence of calf serum will probably increase the number of purification stages required. Recombinant proteins are in many cases present in particles that need to be solubilized and refolded (Chapter 7). In conclusion, not only is it important to discuss the upstream processing in the light of all the protein purification stages, but also to make the necessary decisions to improve the recovery of the protein product early in the process development stages.

The two subprocesses of recovery and purification are discussed in more detail below.

V. PROTEIN RECOVERY

Recovery comprises taking the broth out of the biochemical reactor system (e.g., a fermenter, a hollow fiber reactor) and processing it until a cell-free solution is obtained in which the total protein concentration including the product is around 60 to 70 g/liter (Asenjo et al., 1989; Pharmacia, 1983).

Cell separation or harvesting is always required. The variety of equipment found in industrial practice is not very large (centrifuge, rotary vacuum filter, membrane filtration) and the decision depends on the microbial source, equipment availability, equipment efficiency, and economics. If the product of interest is secreted (extracellular), then the liquid part is kept (Fig. 1); if the product is intracellular, the solid fraction of the harvesting is kept (Fig. 2). When a mammalian cell culture is used, the product is usually extracellular. Typical harvesting operations used are centrifugation (mainly for yeast, but also for mammalian cells and bacteria), rotary vacuum filtration (mainly for fungi), and microporous filtration (for bacteria, yeast, mammalian cells, and also fungi).

Cell disruption is required when the product is intracellular. The equipment is selected mainly on the basis of the microbial source, as each type of microbe presents particular resistance to disruption. The choice of disruption technique determines the size of the resulting debris, which in turn has an influence on subsequent operations. Typical operations used are pressure homogenization (most bacteria including Escherichia coli and yeast) and bead milling (gram-positive bacteria and specific yeast applications). Mechanical disruption releases nucleic acids which need to be precipitated. There is

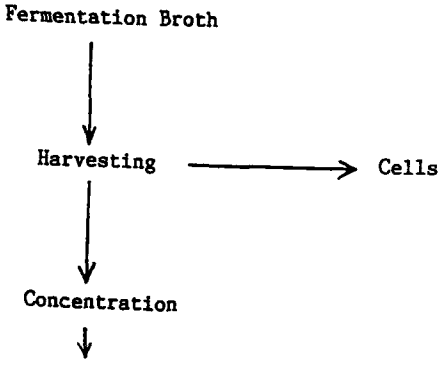

Figure 1 Recovery subprocess of extracellular product (yeast, mammalian, bacterial).

one standard method to achieve nucleic acid precipitation (use of polyehtyleneimine). Separation of cell debris from the proteins in solution must be undertaken once the cells are disrupted. As a result of this step, the product will be in a solution with other proteins but without solids.

If the intracellular product is manufactured in E. coli, high expression of heterologous proteins will usually accumulate in the form of insoluble inclusion bodies. This makes necessary the processing of the inclusion bodies into the native protein by denaturing and

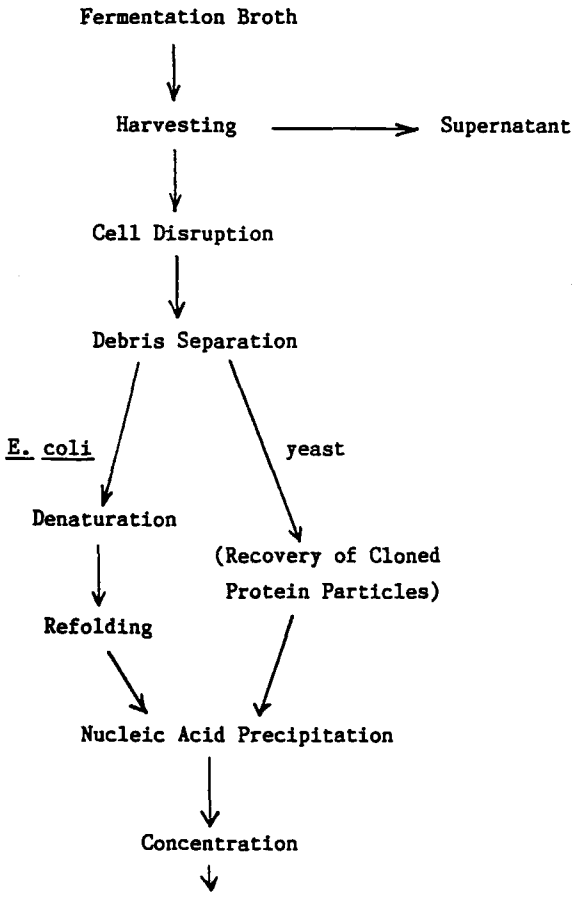

Figure 2 Recovery subprocess of intracellular product (E. coli, yeast).

refolding. If the intracellular product is manufactured in yeast, in many instances the protein is present in homogeneous particulate form, typically 30 to 60-nm particles such as viruslike particles (VLPs). Although the processing of intracellular particulate recombinant proteins is an important aspect of downstream process, there are not many satisfactory methods for large-scale separation, denaturation, and refolding of the particulate proteins. Recent developments in the use of reverse micelles for protein refolding and of two-phase aqueous systems for separation of VLPs from yeast homogenates appear particularly attractive.

Concentration is usually required when the protein concentration of the harvested, disrupted, and separated stream is below 60–70 g/liter. With some proteins it is very difficult to obtain higher concentrations without a serious increase in viscosity, which would then impose poor transport characteristics on the system. If a membrane (ultrafiltration) is used for concentration, the resulting flux characteristics determines the highest possible concentration that could be obtained from the operation. If, at the point where flux has dropped below an acceptable limit, the concentration is below 60 g/liter, then the proteins can be precipitated (e.g., with ammonium sulphate) to increase the final concentration.

VI. PROTEIN PURIFICATION

At this point one if faced with a broth of proteins and some other components such as lipids and/or wall or other polysaccharides, salts, and water in a concentrated protein solution. After considerably reducing the solution volume in the previous concentration step, the total protein content suitable for chromatographic purification is around 60–70 g/liter (Pharmacia, 1983; Asenjo and Patrick, 1990; Asenjo et al., 1989). Here, there will be a number of alternative combinations of purification processes (Table 1). Computer methods used in classic chemical engineering process design and process synthesis as well as expert systems will be useful for optimizing protein purification sequences, as will be discussed in more detail in Chapter 17 of this book. For the recovery, resolution, and purification of a single protein, ideally one would like one step to extract almost 100% of the protein from this mixture with no contaminants. As this is virtually impossible, one will probably need two, or in some cases three of four, stages to achieve the final purity required for the particular application.

Since most of the excess water has been extracted, one would try to use a purification step of extremely high resolution in order to minimize the number of stages used and hence maximize yield. However, this may not be possible in many cases at this stage as some of the contaminants still present may produce fouling of the

Table 1 Chromatographic Operations for Large-Scale Purification of Proteins

Physicochemical property	Operation	Characteristic	Use
Van der Waals forces, H bonds, polarities, dipole moments	Adsorption	Good to high resolution, good capacity, good to high speed	Sorption from crude feedstocks, fractionation
Charge (titration curve)	Ion exchange	High resolution, high speed, high capacity	Initial sorption, fractionation
Surface hydrophobicity	Hydrophobic interaction	Good resolution, speed and capacity can be high	Partial fractionation (when sample at high ionic strength)
Biological affinity	Affinity chromatography	Excellent resolution, high speed and high capacity	Fractionation, adsorption from feedstocks
Isoelectric point	Chromatofocusing	Very high resolution, high speed and very high capacity (limited by size)	Fractionation
Molecular size	Gel filtration	Moderate resolution, low capacity, excellent for desalting	Desalting, end polishing, solvent removal
Hydrophilic and hydrophobic interactions	Reversed-phase liquid chromatography	Excellent resolution, intermediate capacity	Fractionation

Source: Asenjo and Patrick, 1990.